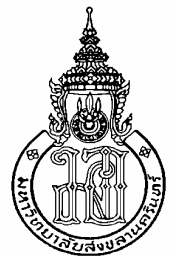

Effect of Hemoglobin, Galangal and Lemon Grass Extracts on Lipid Oxidation in Fish Muscle

Chaiwut Buaniaw

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Food Technology

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ชื่อวิทยานิพนธ์	ผลของฮีโมโกลบิน สารสกัดจากข่าและตะไคร้ต่อการเกิดออกซิเดชันของไขมันในเนื้อปลา
ผู้เขียน	นายชัยวุฒิ บัวเนี่ยว
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ปีการศึกษา	2550

บทคัดย่อ

เนื้อปลาโอมีองค์ประกอบทางเคมีดังนี้ คือ ความชื้น ไขมัน โปรตีน และเถ้า ร้อยละ 78.35±0.39, 4.57±0.49, 14.79±1.18 และ 1.14±0.01 ตามลำดับ ส่วนเนื้อปลากะพงขาวมีองค์ประกอบดังกล่าวร้อยละ 78.37±0.22, 3.87±0.39, 17.11±0.10 และ 1.09±0.01 ตามลำดับ นอกจากนี้ปลาโอ และปลากะพงขาวมีปริมาณของเหล็กทั้งหมดเท่ากับ 5.979±0.023 และ 3.268±0.009 mg/100g โดยมีปริมาณเหล็กในรูปของฮีมอยู่ 4.221±0.215 และ 1.207±0.052 mg/100g ตามลำดับ ส่วนของโปรตีนฮีมที่สกัดได้มีอยู่ 480.40±2.20และ 378.70±1.60 mg/100g ตามลำดับ ซึ่งฮีโมโกลบินของปลากะพงขาวแสดงสมบัติการดูดกลืนแสงเช่นเดียวกับที่พบในสัตว์ชนิดอื่น ๆ โดยแสดงค่าการดูดกลืนแสงสูงสุดที่ 412, 545 และ 565 nm

จากการศึกษาผลของพีเอช และเอทีพีต่อสมบัติการปลดปล่อยออกซิเจนออกจากโมเลกุลฮีโมโกลบินของปลากะพงขาว พบว่าเมื่อระดับพีเอชของระบบลดลงจาก 7.5 เป็น 5.5 มีผลทำให้ปริมาณดีออกซีฮีโมโกลบินเพิ่มขึ้นในลักษณะเส้นโค้งรูปตัว S โดยที่ระดับพีเอช 6.2 เป็นจุดที่มีการเปลี่ยนแปลงรวดเร็วที่สุด ในขณะที่สารเอทีพีมีผลเร่งการเกิดดิออกซีจีเนชันของฮีโมโกลบินน้อยกว่าผลของพีเอช โดยฮีโมโกลบินแสดงสมบัติเป็นสารโปรออกซิแดนท์ในระบบของเนื้อปลาบดที่มีพีเอชเท่ากับ 6.2 แต่กลับไม่มีผลเมื่อพีเอชเท่ากับ 7.0 เมื่อพิจารณาจากระยะเวลาที่ค่า TBARS มากกว่า 7 mg MDA/kg แม้ว่าการล้างเนื้อปลาบดเป็นปัจจัยหนึ่งที่มีผลชะลอการเกิดออกซิเดชันของไขมัน หากเนื้อปลาบดที่ผ่านการล้างอยู่ในสภาวะเร่งที่พีเอช 6.2 และเติมฮีโมโกลบินลงไป 5.8 μmole/kg มีผลทำให้อัตราการเกิดออกซิเดชันของไขมันรวดเร็วยิ่งขึ้น ส่วนในกรณีที่ระดับพีเอชเท่ากับ 7.0 ค่า TBARS เพิ่มขึ้นช้ากว่าชุดควบคุม แสดงว่าพีเอชของระบบเป็นปัจจัยสำคัญที่มีผลต่อการสมบัติโปรออกซิแดนท์ของฮีโมโกลบิน

ปลากะพงขาวในระยะการตายที่ 2 10 และ 48 ชั่วโมง มีค่าแรงตึง พีเอช และ TBARS แตกต่างกัน ($p<0.05$) แต่ค่าลิปิดไฮโดรเปอร์ออกไซด์ (LHP) ไม่แตกต่างกัน ($p<0.05$) และพบว่าการเกิดออกซิเดชันในเนื้อปลาบดจากปลาระยะที่ 3 มีค่า LHP และ TBARS เพิ่มขึ้นรวดเร็ว

มากที่สุด นอกจากนี้ในเนื้อปลาบดที่ผ่านการล้างน้ำของระยะที่ 3 มีค่า TBARS เพิ่มขึ้นมากเกิน 7 mg MDA/kg ภายใน 30 และ 54 ชั่วโมง ที่พีเอช 6.0 และ 6.2 ตามลำดับ แต่ตัวอย่างปลากะพงขาวในระยะที่แรก และ 2 ยังคงมีค่า TBARS ไม่เกินค่าดังกล่าวแม้เก็บไว้นานภายใน 60 ชั่วโมง อย่างไรก็ตามพบว่าหากมีการเติมฮีโมโกลบินลงไปในเนื้อปลาบดที่ผ่านการล้าง มีผลทำให้ทั้ง ค่า LHP และ TBARS เพิ่มขึ้นเร็วอย่างมีนัยสำคัญทั้งสามระยะ เมื่อเปรียบเทียบกับเนื้อปลาที่ไม่มีการเติมฮีโมโกลบินลงไป

การสกัดข่าและตะไคร้ด้วยสารละลายเอธานอลร้อยละ 50 มีผลให้ได้ปริมาณสารประกอบพอลีฟีนอลมากที่สุด ($P<0.05$) ตามด้วยสกัดด้วยสารละลายเอธานอลร้อยละ 75 35 0 (น้ำกลั่น) และ95 ตามลำดับ นอกจากนี้ยังพบว่าปริมาณสารประกอบโพลีฟีนอลที่สกัดได้จากข่ามากกว่าที่ได้จากตะไคร้ นอกจากนี้สารสกัดจากข่าด้วยสารละลายเอธานอลร้อยละ 95 (GE95) มีความสามารถในการจับอนุมูล DPPH ได้ดีที่สุด เมื่อความมีขั้วของตัวทำละลายที่ใช้ในการสกัดเพิ่มขึ้นพบว่ามีความสามารถจับอนุมูล DPPH เพิ่มขึ้น ความสามารถในการจับเหล็ก (Fe^{2+}) ของสารสกัดจากข่าดีกว่าสารสกัดจากตะไคร้ แต่ความสามารถในการจับโลหะเหล็กมีความสอดคล้องกับการลดลงความมีขั้วของสารตัวทำละลายที่ใช้ในการสกัดสารจากตะไคร้ แต่ไม่มีความสอดคล้องกันในกรณีของสารสกัดจากข่า (R^2= 0.9706 และ 0.0709 ตามลำดับ) เมื่อนำสารสกัดเข้มข้น 0.02 mg GAE (gallic acid equivalent) เติมลงในระบบเนื้อปลาบดที่ผ่านการล้างและเติมฮีโมโกลบินในสภาวะเร่งที่พีเอช 6.2 พบว่า GE95 และตะไคร้ด้วยสารละลายเอธานอล 95 (LE95) สามารถชะลอการเพิ่มขึ้นของค่า TBARS ได้ถึง 7.5 เท่าของชุดควบคุม เมื่อความมีขั้วของสารสกัดลดลงมีผลเพิ่มความสามารถของสารสกัด ในการชะลอการเกิดออกซิชันของไขมันในเนื้อปลาบดที่มีฮีโมโกลบินเป็นตัวเร่ง (R^2 = 0.9906) แต่ LE95 มีกลิ่นที่ส่งผลระบบเนื้อปลาบด นอกจากนี้เมื่อนำ GE95 ไปทดสอบในระบบสารละลายที่มีฮีโมโกลบินในอัตราส่วน 1 ต่อ 1 mole พบว่าสาร GE95 มีผลชะลอการเกิดดีออกซีฮีโมโกลบิน ดังนั้น GE95 จึงเป็นสารสกัดที่ได้นำไปใช้ในการศึกษาการล้างชิ้นเนื้อปลา

ประสิทธิภาพการล้างเนื้อปลาที่มีระดับความรุนแรงของการออกซิเดชันที่แตกต่างกันซึ่งได้ทดลองในระบบเนื้อปลากะพงขาว(มีคุณภาพความสดดีกว่า) และปลาโอ(มีคุณภาพความสดต่ำกว่า) โดยใช้สารลาย GE95 2% GAE พบว่า การเปลี่ยนแปลงของค่าพีเอช LHP, TBARS และค่าสี (L^* a^* และ b^*) ของเนื้อปลาที่ผ่านการล้างด้วยสารสกัดมีการเปลี่ยนแปลงน้อยกว่าชุดควบคุม โดยเฉพาะในเนื้อปลาโอ แต่กลับพบว่าค่า LHP และ TBARS ไม่มีความแตกต่างกันในปลากะพงขาวในตลอดการเก็บรักษา 15 วัน

Thesis Title	Effect of Hemoglobin, Galangal and Lemon Grass Extracts on Lipid Oxidation in Fish Muscle
Author	Mr. Chaiwut Buaniaw
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ABSTRACT

Dorsal flesh of tongol tuna contained 78.35±0.39% moisture, 14.79±1.18% protein, 4.57±0.49 % lipid and 1.14±0.01% ash, whereas the sea bass dorsal flesh had those proximate components of 78.37±0.22, 17.11±0.10, 3.87±0.39 and 1.09±0.01%, respectively. Additionally, the total iron content of tongol tuna and sea bass was 5.98±.02 and 3.27±0.01 mg/100g; heme-iron was 4.22±0.22 and 1.21±0.22 mg/100g, and hemoglobin content was 0.48±0.01 and 0.38±0.01 mg/100g, respectively. The sea bass hemoglobin showed three peaks at 412 nm (Soret region), 545 and 575 nm (oxygenation indicator).

Effect of pH and ATP on deoxygenation of hemoglobin in sea bass hemolysate was examined. It was found that a decrease of pH from 7.5 to 5.5 lowered relative amount of deoxyhemoglobin (DeoxyHb) in sigmoidal fashion. The rate of (de)oxygenation occurred rapidly at pH 6.2. ATP stimulated the deoxygenation at pH 7.0. Moreover, pH affected the pro-oxidative property of hemoglobin in minced sea bass, especially at pH 6.2 the TBARS elevated to 7 mg MDA/kg faster than that of pH 7.0 condition. Washing of sea bass muscle mince delayed the development of lipid oxidation. Whereas, the addition of hemoglobin (5.8 μmole/kg) to washed and unwashed mince at pH 6.2 or unadjusted pH accelerated lipid oxidation, but did not at pH 7.0. The results suggested that DeoxyHb was a potent catalyst of lipid oxidation in fish muscle.

The post mortem stage I, II and III (2, 10 and 48 hr after death) of sea bass showed differences in firmness, pH and TBARS values, but not the LHP. The post mortem stages affected to the lipid oxidation either in the washed or unwashed minces, regardless to the addition of hemoglobin and pH adjustment of the minces. The minces prepared from the stage III fish showed the fastest development rate of lipid oxidation followed by that of the stage II and I fish, respectively. Moreover, the washing did not show an effectiveness on inhibition of lipid oxidation in washed mince of stage III fish, the TBARS increased to 7 mg MDA/kg within 30 and 54 hr at pH 6.0

(unadjusted pH) and 6.2, respectively. The results suggested that the sea bass deoxyhemoglobin could be a potent pro-oxidant especially at low pH.

Galangal and lemon grass were extracted with ethanol and used for evaluation their antioxidative properties. The polyphenolic compound content was the highest yield at 50% ethanolic extract and followed by 75, 35, 0 (distilled water) and 95 %, respectively. Most of galangal extracts showed higher content of phenolic content than lemon grass extract, but at 0 and 95% ethanolic extracts was not difference. However, 95% ethanolic extract of galangal (GE95) showed the lowest IC_{50} (11.67$\pm$0.29 mg/ml) of DPPH radical scavenging activity. The better correlation between the ethanol concentration, [EtOH], and the DPPH radical scavenging activity was found in galangal (R^2 = 0.8823, $p<0.05$) than in lemon grass (R^2 = 0.6167, $p<0.05$). Interestingly, the iron (Fe^{2+}) chelation activity of galangal was very low correlated to [EtOH] (R^2 = 0.0709, $P<0.05$), but highly chelation activity. Whereas, the iron chelation activity of lemon grass depended on the [EtOH] (R^2 = 0.9706, $p<0.05$).

The addition of the extracts (0.02 mg gallic acid equivalent, GEA) into washed sea bass mince with an accelerated condition at pH 6.2 plus 5.8 μmole hemoglobin/kg muscles were evaluated by the development of TBARS values. GE95 and 95% ethanolic extract of lemon grass (LE95) could retard the TBARS more than 7.5 times compared with control sample. Interestingly, a good correlation between the [EtOH] in extraction with the inhibitory activity of lipid oxidation in accelerated minced system (R^2 = 0.9906, $p<0.05$) was observed. However, the LE95 caused a bad smell in mince. Moreover, GE95 showed the reduction of the DeoxyHb formation, just after mixing with the hemoglobin solution in a ratio of 1:1 mole at pH 6.5. Therefore, GE95 was chosen for the next experiment.

Tongol tuna and sea bass slices expressed as low and high freshness flesh, respectively. The effectiveness of fish slices washing in the diluted solution of GE95 (0.2 % w/v GAE) or distilled water were studied. It was found that the smaller changes of parameter included LHP, TBARS and color (L^*, a^* and b^* values) in GE95 treated sample through 15 day of storage at 4°C. Soaking in GE95 was more effective in reducing LHP and TBARS of tongol tuna than in sea bass. The results suggested that galangal extracts showed good antioxidant activity.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

It is well recognized that oxidation of the lipid fraction of fish muscle is a major cause of quality deterioration in fatty fish due to the high degree of fatty acid unsaturation (Morrissey and Tichivangana, 1985). However, a recent data suggested that rancid odor and extensive lipid oxidation could be developed in the system having marginal amount of polyunsaturated lipid (Sohn *et al.*, 2005). Blood and its components are being scrutinized as great pro-oxidants. Blood is known to cause deterioration in muscle foods. For this reason farm animals are generally bled as much as possible when they are slaughter. Fish are often not bled when they die. In fact, they may have been dead for some time before they are processed. Bleeding of mass wild fish, especially small fish is not practicable and not cost effective. Several attempts to bleed farm fish or wild big fish revealed that the residual blood level in the muscle of bled fish was substantial. The beneficial effect of bleeding on quality of bled fish was varied. Tretsven and Patten (1981) reported that cutting arteries of rainbow trout reduced the amount of rancidity development after frozen storage for 8 months. As well as, bleeding significantly reduced rancidity in minced trout muscle, minced mackerel light muscle, and intact mackerel dark muscle but not minced mackerel dark muscle stored at 2°C (Richards and Hultin, 2002). On the other hand, Porter *et al.* (1992) found that bleeding did not decrease the rate of lipid oxidation between groups of bled and unbled sockey salmon as measured by TBARS or loss of unsaturated fatty acids. Various factors including hemoglobin concentration, types of hemoglobin, plasma volume, and erythrocyte integrity on blood-mediated lipid oxidation in fish muscle were proposed (Richards and Hultin, 2002).

Richards and Hultin (2002) found that development of lipid oxidation in washed mince cod associated with an increase the cod deoxyhemoglobin. Factors increased amount of the reduced hemoglobin such as pH lower than 7.6 or an exposure to ATP at neutral pH were, thus, found to shorten the lag time before the development of rancidity. Similar strong oxidizing effect of

hemoglobin at pH 6.5 or below has been reported (Binotti *et al.,* 1971; Everese and Hsia, 1997). This adverse effect of low pH was proposed to be related to the well known Bohr effect (Riggs, 1970). Apart from pH, organic phosphates are capable of forming cross links between the tetrameric subunits of hemoglobin by binding to amino acid at the entrance to the central cavity and thereby decreasing oxygen affinity. Thus, peculiar complaint of an intense paintiness in the fillet of the lean, white-flesh fish like sea bass may be associated with an increase of pro-oxidative activity of its hemoglobin. This is also supported in part by the fact that postmortem pH of sea bass is around 6.5 (Buaneow *et al.*, 2008). Moreover, heading and cutting of fish is normally done just after fish killing, thus high amount of ATP residue in such pre-rigor fish is expected.

Richards *et al*. (1998) found that unwashed mackerel fillet underwent rapidly oxidative deterioration if the blood was not completely removed from the surface. Extracts of the cut surface tissue indicated that the rate of oxidation was related to the heme content in the extract. In comparing the storage stability of frozen fish filleted underwater has a significant longer shelf-life compared with frozen fish filleted in air. The contribution by the blood thus occurred quickly and application of antioxidant in washing water is promising (Richards *et al.*, 1998).

Due to increasing awareness on safety of food additives like antioxidants, there is a great interest in obtaining and utilizing the antioxidants from natural sources. Antioxidant activity of extracts obtained from spices and herbs daily used for cooking food have been reported (Botsoglou *et al.*, 2002; Ahn and Nam, 2004). Several extracts are introduced to the market and suggested to use in food systems. Galangal and lemon grass are generally Thai cooking food ingredients. Many researchers found that galangal rhizome and lemon grass have antioxidative, antimycotic, antimicrobials and anticancer properties (Wang *et al.*, 1997; Cheah and Abu-Hasim, 2000b; Javanmardi *et al.*, 2003). Extracts of galangal and lemon grass inhibited effectively the development of lipid oxidation in food products (Wang *et al.*, 1997; Cheah and Abu-Hasim, 2000b; Javanmardi *et al.*, 2003). Therefore this research might be a schematic of the role of hemoglobin on lipid oxidation and using of galangal and lemon grass extracts to inhibit lipid oxidation in fish muscle.

Literature Review

1. Fish

Fish is an excellent protein source for human consumption, it consists of a high content of hydrosoluble and liposoluble vitamins, minerals and polyunsaturated fatty acids (PUFAs). Interestingly, omega-3 fatty acids, found mainly in fat-rich fish such as salmon, mackerel, herring, and sardines have benefit on human health. The American Heart Association (AHA) advocates consumption of fatty fish at least twice a week, as a safe and effective way to obtain the heart health benefits of omega-3 fatty acids (Kris-Etherton *et al.*, 2002; Hooper *et al.*, 2005). However the enrichment of unsaturated fatty acids is a major factor of lipid oxidation in fish.

Sea bass (*Lates calcarifer* Blotch), Asian sea bass, Giant sea perch, Cock-up, or Barramundi is an economically important food fish and sport fish in the tropical and subtropical areas of Western Pacific and Indian Ocean countries, including India, Myanmar, Sri Lanka, Bangladesh, Malay Peninsula, Java, Celebes, Philippines, Papua New Guinea, Northern Australia, Southern China, and Taiwan (Boonyaratpalin, 1994). The sea bass is an euryhaline fish, meaning it can live in both fresh and salt water (Shipp, 1996).

Tongol tuna (*Thunnus tongol*) and tuna – like species are important fish species due to their high global economic value and their prevalence in international trade for canning and sashimi. Tuna is the largest pelagic fish that prevails in the (sub) tropics (Al-Abdessalaam, 1995). It is warm blood fish and keeps its body at higher temperature than the surrounding water (King *et al.*, 1995). The average length and weight of these fish are 47.3 cm and 1.74 kg, respectively (Mazorra-Manzano *et al.*, 2000).

Sea bass and tongol tuna are different in behavior, activity and attribute. Sea bass is a white flesh fish that is not strong swimmer and do not regularly travel over long distance, whereas, tongol tuna has more habitual activity in swimming for surviving and chasing. It might be the regular activities which are determining their flesh components that necessary for functions of muscle (Love *et al.*, 1977).

2. Fish post-mortem changes

In living muscles the fuels for producing the ATP are either free fatty acids, glucose from the blood, or glycogen which is stored within the muscle fibers. In the fasting state, free fatty acids derived from the break down of triglyceride in the fat depots of the body are metabolized. Glycogen is mobilized only when the rate of breakdown of fatty acids and glucose cannot provide sufficient energy for contraction of muscles. However, oxygen is a major necessary for the production of ATP because it can provide 36-37 ATP per aerobic metabolic cycle. Glycogen and glucose are breakdown by the same process. This involves the operation of these interrelated processes: glycolysis, oxidative decarboxylation and oxidative phosphorylation (Verhees *et al.*, 2003).

The biochemical reactions of live fish are changed after death. At that point, the respiratory system is interrupted and the blood circulatory system failed. Therefore, the oxygen supplying is not available for cellular respiration and the production of energy is restricted. Under the lacking of oxygen or anaerobic conditions, any subsequent metabolism must be generated through the pathway of creatine phosphate and glycolysis, since oxidative decarboxylation and phosphorylation will no longer operate. From the operation of glycolytic metabolism, ATP, might be synthesized and also the pyruvate is reduced by NADH to form lactic acid. Instead, for every glycogen moiety, equivalent to glucose molecule, two lactic acid molecules are produced. As glycogen is broken down but lactic acid accumulates. Because this is not removed by the blood system then the muscle gradually acidifies. The changes in post mortem pH in such animals are related to the production of lactic acid form glycogen. In general, fish muscle contains a relatively low level of glycogen compared to mammals; thus far less lactic acid is generated after death. Also, the nutritional status of the fish and the amount of stress and exercise encountered before death will have a dramatic effect on the levels of stored glycogen and consequently on the ultimate post mortem pH (Verhees *et al.*, 2003).

The biochemical process occurred during post mortem period can be divided into three steps: pre-rigor, rigor and post-rigor. Rigor or rigor-mortis describes the contractile process stimulated by lacking of ATP resulting in both shortening and rigidity of muscles that persists for several hours or days, depending on a number of factors. The onset of rigor-mortis varies from 3 to 6 hours in smaller animals such as poultry, and 24 to 36 hours in larger animals (Gill, 2005).

Different muscles contain different proportions of the different fiber types and this determines the macroscopic color of the muscle.

In a recent study of Japanese researchers (Chiba *et al.*, 1991) found that only minutes of pre-capture stress fish resulted in a decrease of 0.50 pH units in 3 hours as compared to non-struggling fish whose pH dropped only 0.10 units in the same time period. The level of creatine phosphate then falls as it is used to regenerate ATP from ADP. Eventually however, the supply of generated ATP fails. Rigor mortis occurs when the ATP level falls below the low level (~5 mmol kg^{-1}) required maintaining relaxation. When this happens, the actin and myosin molecules of thin and dark filaments combine irreversibly to form actomyosin and extensibility of muscle is lost. A Cross-bridge form permanently and there is in effect a very weak contraction. Each muscle fiber go into rigor very quickly once ATP is depleted, but the variation between individual fibers leads to a more gradual development of stiffness in the whole muscle as more and more fibres become inextensible. The time of onset of rigor will obviously relate to factors affecting the level of glycogen and creation phosphate at death and the rate of post mortem muscle metabolism (Warris, 2000).

3. Lipid oxidation

Free radicals can be defined as any chemical species having one or more unpaired electrons (Hamilton *et al.*, 1997). Such a wide definition covers the hydrogen atom, transition metal atoms and the oxygen molecule itself, with its two unpaired electrons, acts like a biradical. One of the major areas in which carbon free radicals and oxygen free radicals involved is in the oxidation of lipids (Carlsen *et al.*, 2005). Lipid is any derivatives of a fatty acid which covers a host of compounds that associated with triacylglycerol and ester with free fatty acids attached to one alcohol-glycerol (Vincent *et al.*, 2002b). The lipid containing large amount of polyunsaturated fatty acid can react very quickly with at atmospheric oxygen. Then the lipid oxidation is activated through the one of three pathways as presented in Figure1 (Carlsen *et al.*, 2005).

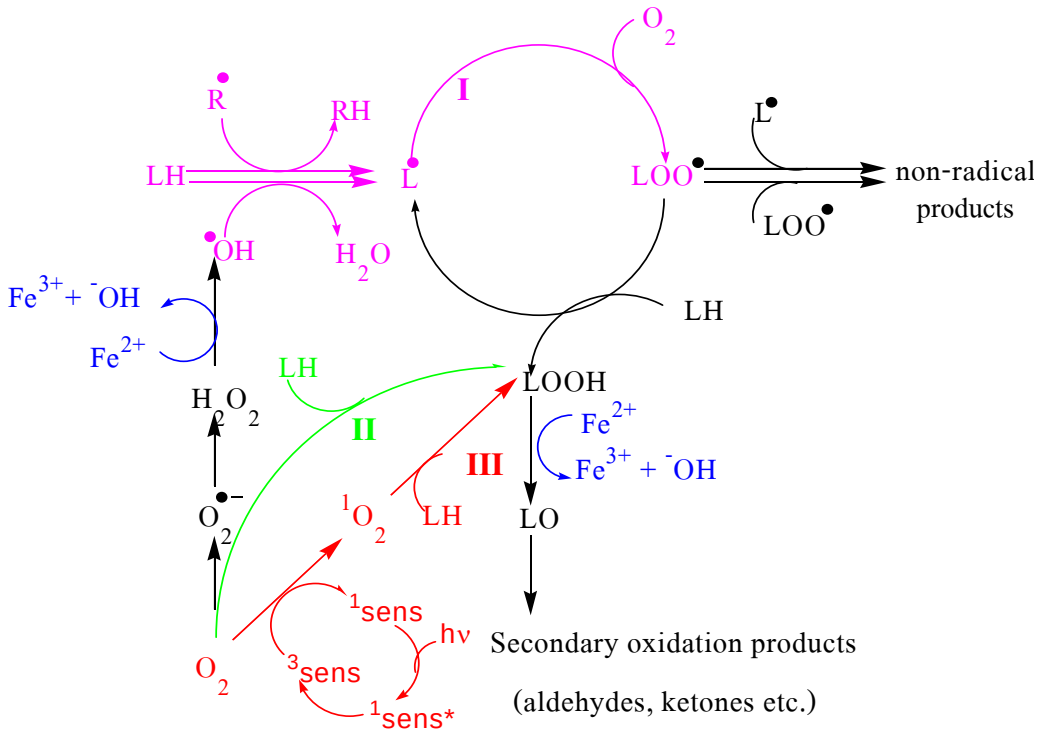


Figure 1 Three different pathways of lipid oxidation

Source: Carlsen *et al.* (2005)

3.1 Pathway I. Autoxidation

The autoxidation or thermal lipid oxidation occurs when the atmospheric air interacts with free radical intermediates. The autoxidation of oils and fats involves catalysis by traces of transition metal compounds, and involves biological fluids like milk and blood, and by enzymes like the molybdenum containing xanthine oxidase, copper-zinc containing superoxide dismutase, and the iron containing peroxidases (Carlsen *et al.*, 2005).

A mechanism for the autoxidation of a methylene-interrupted unsaturated system (e.g. linoleate) was proposed by Holman (1954). The various reactions which may occur are presented in Figure 1. The initiation step called deprotonation (***step I***) involves the abstraction of a hydrogen atom (H) from the methylenic carbon atom adjacent to a double bond. Although, these reaction result have not been fully elucidated. It is found that the reaction produces several free radicals, e.g. peroxy ROO°, alkoxy RO° and alkyl R°. These radicals can rapidly react with oxygen according to equation 1 with the reaction constant value of 10^9 $1 \cdot mol^{-1}$ s^{-1}. Catalysts are necessary;

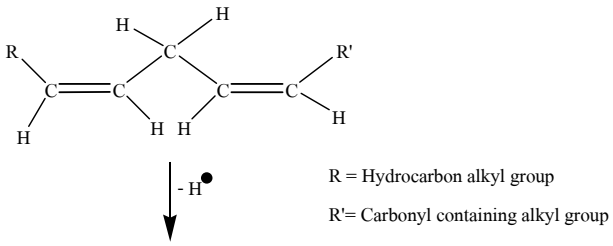
otherwise the reaction would be too slow to be significant. The reaction is catalyzed by several catalysts (trace metals, oxygen, light, etc.) or enzymes (lipoxidases). The free radical (***step II***) is resonance hybridization; the two extreme forms are shown as ***step III***. Molecular oxygen added to the resonating radical, predominantly at the ends, to yield two types of hydroperoxy radicals (***step IV***). The latter free radicals (***step IV***) can accept hydrogen atoms from other molecules (linoleate) to become isomeric conjugate *cis, tran*-hydroperoxides, and propagate the chain reaction (Aurand and Woods, 1973).

If the peroxy radical is formed it can attack another lipid molecule or the starting molecule to remove a hydrogen atom. In this way a hydroperoxide and another free radical are produced (***step V***). This hydroperoxide is the intermediate mentioned earlier. The reason why these changes are so destructive is that the hydroperoxide can break down to give two radicals (either an alkoxide or a hydroxyl) or it can give a peroxy free radical, hydroxy free radical and water. These branching steps lead to proliferation of free radicals which may go back to aid the propagation steps thus the reaction becomes autocatalytic. The reactions can be terminated in a low oxygen environment by equation (1) or either in a high oxygen environment by equation (2) and (3). The conversion of Fe^{2+} to Fe^{3+} produces an alkoxide free radical. The Fe^{3+} is also capable of decomposing the hydroperoxide, giving a peroxy free radical and itself being reduced to Fe^{2+}. This reaction cycle results in large quantities of derived lipid (Carlsen *et al.*, 2005).

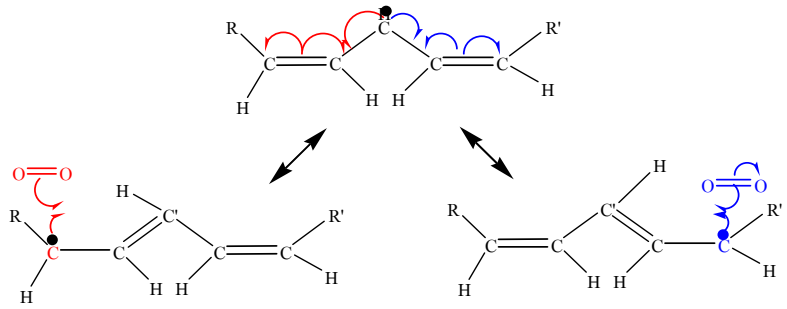
3.2 Pathway II: Enzymatic oxidation

Lipoxygenases are non-heme-iron enzymes catalyzing deoxygenation of polyunsaturated fatty acids and are widespread in the animal and plant kingdom (Liu *et al.*, 1998). Glycerides, glycolipids and phospholipids are hydrolysed by lipases to form free fatty acids. Lipoxygenase then reacts with free fatty acid much more readily than with bound lipid and produces a hydroperoxide. Hydroperoxide lyases then convert this molecule to aldehydes, ketols, divinyl ether fatty acids, epoxy hydroxy fatty acids and trihydroxy fatty acids.

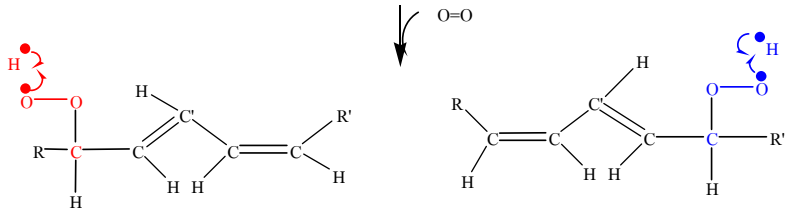
I. Deprotonation



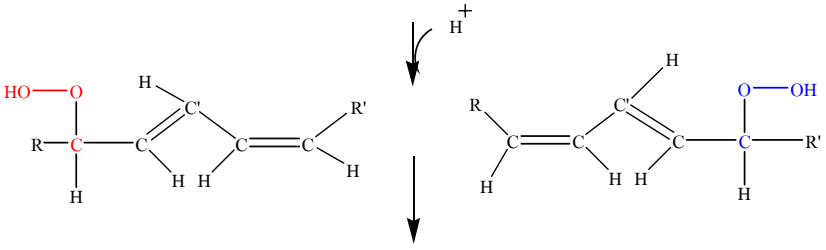
II. Formation of free radicals



III. Oxygenation



IV. Protonation



V. Termination

$$2\ R^{\circ} \rightarrow R \quad (1)$$

$$R^{\circ} + ROO^{\circ} \rightarrow ROOR \quad (2)$$

$$ROO^{\circ} + ROO^{\circ} \rightarrow ROOH + O_2 \quad (3)$$

Figure 2 Autoxidation of polyunsaturated lipid (Where, R° = alkoxyl radical, ROO° = Peroxy radicals, ROOH =hydroperoxides)

Source: Aurand and Wood (1973)

3.3 Pathway III: Photo oxidation

The photooxidation involves a photosensitizer like riboflavin or chlorophyll for formation of singlet-oxygen or for direct formation of radicals (Skibsted *et al.*, 1998). It is known that light accelerates the lipid autoxidation but direct photo oxidation is unlikely because the unsaturated fatty acid cannot absorb light energy of wavelength < 220 nm. The orbital of oxygen shows that there are two ways in which electrons can be inserted. The triplet state has two unpaired electrons whilst singlet oxygen has electrons paired and is not a free radical. The fact that it has a vacant orbital means that, it is looking for electrons from electrophilic molecules. So it can react with the double bonds of unsaturated fatty acids. For these oxidations to occur, sensitizer molecules such as chlorophyll and porphyrin must be existed (Miskoski *et al.*, 1994).

3.4 Formation of superoxide and decomposition of (lipid) hydroperoxides

Superoxide ($^{\circ}O_2^-$) can be generated via a number of ways (equation 4): for example, some complexes of iron e.g. heme, generate superoxide (equation 5); reducing agents such as ascorbate (AsH^-) contribute to the redox cycle (equation 6); Xanthine oxidase (X) can also generate the superoxide radical (equation 7). Superoxides react with many molecules but their rates are low (Hippeli and Elstner, 1991).

$$O_2 + Fe\ (II) \longrightarrow {}^{\circ}O_2^- + Fe\ (III) \quad (4)$$

$$O_2 + Heme\ (II) \longrightarrow {}^{\circ}O_2^- + Heme\ (III) \quad (5)$$

$$Fe\ (III) + AsH^- \longrightarrow Fe\ (II) + {}^{\circ}As^- + H^+ \quad (6)$$

$$X + O_2 \longrightarrow urate + n{}^{\circ}O_2^- + (n\text{-}1)\ H_2O_2 \quad (7)$$

The formation of hydroperoxide (the primary oxidation product) renders the molecule somewhat unstable is further cleaved oxidatively to yield peroxyl radicals or reductively to yield alkoxyl radicals by metal like-iron. Both alkoxyl and peroxyl radicals may initiate new reaction chains, while alkoxyl radicals are mostly further cleaved to peroxide under go readily decomposition to form short chain carboxylic compounds, alcohols, aldehydes, ketones, and alkanes (Hamilton *et al.*, 1997). These secondary oxidation products are responsible for the development of broad spectrum of (off-) odors, (off-) flavors in oxidized fat. Hydroperoxides are

relatively unstable at or above 80°C, therefore variety of the end products may be affected by different reaction temperatures (Carlsen *et al.*, 2005).

As the lipid is oxidized, the concentration of unsaturated lipid falls and the amount of hydroperoxide increases to a maximum, but it might be fall subsequently. So the monitoring of oxidation mainly based on peroxide value (PV) somewhat can occur confusing. The volatile products, which are the derived lipids, then rise slowly. The types of derived lipid depend on the parent lipid as schemed in Figure 3 (Hamilton *et al.*, 1997).

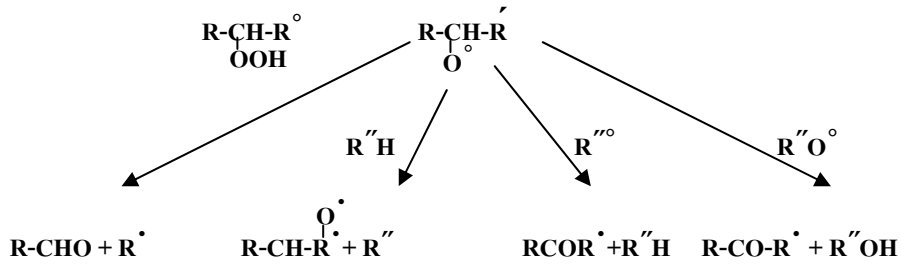


Figure 3 Possible paths of derived lipids formation (where R = side chain group, $R^{\bullet}$ = alkoxy radical, $O^{\bullet}$ = oxygen radical)

Source: Hamilton *et al.* (1997)

This propagation continues until one of the radicals is removed by reaction with another radical or with an antioxidant (AH) resulting a less active radical (A) (Vincent *et al.*, 2002b). The results of hydroperoxide decomposition are substances which some have unpleasant (rancid) taste and smell. In case of proteins, it may also contribute to modification of muscle proteins by binding with proteins. Rancidity is the derived lipid that gives the off-flavors, whilst used to follow the oxidation by many analytical techniques. Several of the aldehydes can be determined as thiobarbituric acid reactive substances (TBARS) that use for monitoring the MDA (malondialdehydes), a major lipid oxidation product (Hamilton *et al.*, 1997).

4. Hemoglobin

4.1 Structure and functions of hemoglobin

Hemoglobin is the major heme protein of red blood cells and is responsible for the transportation of oxygen to the tissues (Perutz, 1990). Hemoglobin consists of four polypeptide chains, two of α-chains and two of β-chains (Figure 4). The four chains are held together by non-covalent attractions. Each α-chain is in contact with β-chains ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). The conformation of the α- and β-subunits differs only by addition of one helix (sequence in D helix) in the β-subunit. Differences in number and identity of amino aids have been reported in hemoglobin from different sources (Perutz, 1990; Olafsdottir *et al.*, 1997) Capacity of hemoglobin oxygenation influenced from a heme group presents in the nonpolypeptide unit (Shikama, 2006).

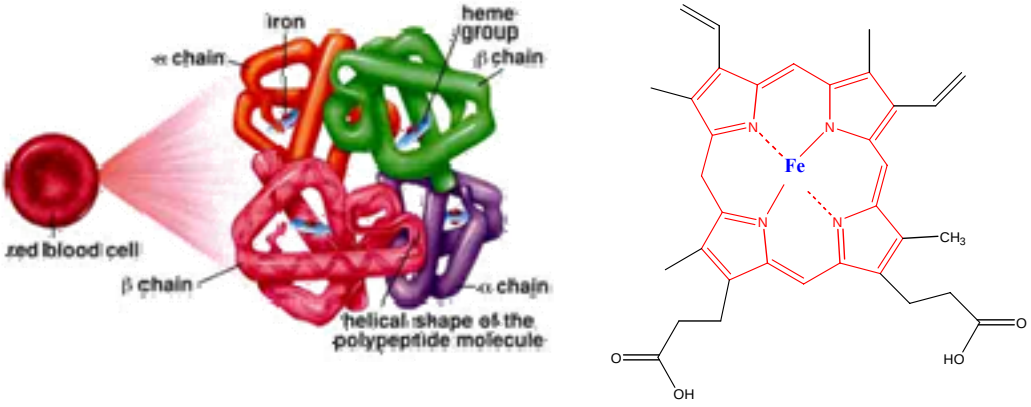


Figure 4 Hemoglobin and porphyrin ring structure

Source: www.biochem.psu.edu.htm/hemo.

The heme buried in hydrophobic pocket of hemoglobin consists of an organic part and an iron atom. The organic part, protoporphyrin, is made up of four pyrrole rings. The four pyrroles are linked by methane bridges to form a tetrapyrrole ring. Four methyl, two vinyl, and two propionate side chains are attached to tetrapyrrole ring. These constituents can be arranged like iron chelating. The ferrous (Fe^{2+}) in the center of heme can reversibly bind to oxygen molecule. From known changes in valency of the heme-iron, one can write the functional cycle of hemoglobin as follows Figure 5 (Shikama, 2006a).

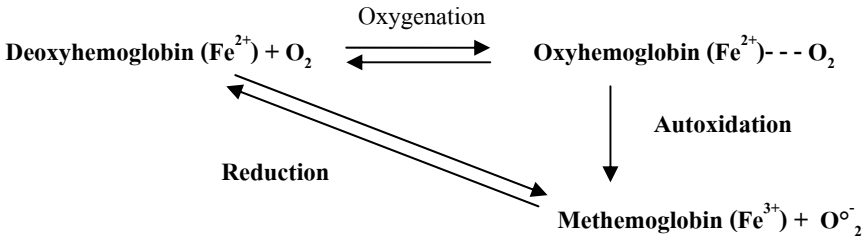


Figure 5 Functional cycle of hemoglobin

Source: Shikama (2006)

From the previous literature, the fishes were classified as three types by fish blood components (Weber and Lykkeboe, 1978). Class I comprising of the anodic hemoglobin that have (normal) Bohr, Root, phosphate, and temperature effects (Weber and Lykkeboe, 1978), class II comprising hemoglobins that consist of both anodal and cathodal hemoglobin, which exhibit high oxygen affinities and small, often reverse Bohr effects and low temperature sensitivities (Binotti *et al.,* 1971; Weber *et al.*, 1976); class III hemoglobin that appear to be sensitive to pH but insensitive to temperature (Rossi-Fanelli *et al.*, 1960).

4.2 Auto-oxidation of hemoglobin

It is possible to propose a mechanism of hemoglobin autoxidation as equations (8) to (13) based on the results and mechanistic considerations of Watkins *et al* (1985).

$$HbO_2 \leftrightarrow Hb + O_2 \quad (8)$$

$$Hb + H^+ + L^- \leftrightarrow Hb\,(H^+)(L^-) \quad (9)$$

$$Hb\,(H^+)(L^-) + O_2 \leftrightarrow MetHb\,(L^-) + (H^+)\,O_2°^- \quad (10)$$

$$HbO_2 + 2H^+ + O_2°^- + H_2O \rightarrow MetHb{\bullet}H_2O + O_2 + H_2O_2 \quad (11)$$

$$Hb + H_2O_2 + H_2O \rightarrow MetHb{\bullet}H_2O + {}^-OH + HO° \quad (12)$$

$$Hb + HO° + H_2O \rightarrow MetHb{\bullet}H_2O + {}^-OH \quad (13)$$

$$4HbO_2 + 2H^+ \rightarrow 4MetHb{\bullet}H_2O + 2H\,O^- + 3O_2 \quad (14)$$

The overall stoichiochemistry of equation 14 indicates that dioxygen undergoes a four electron reduction (one electron from each heme-iron) to convert each oxygen atom to the oxidation state. In the fish muscle system, if heme crevice volume is large, hemoglobin can

autoxidize rapidly in spite of low deoxyhemoglobin content, because a synproportionation reaction of hemoglobin is occurred as equation 15 and 16 (Richards and Dettmann, 2003).

$$\text{DeoxyHb } (Fe^{2+}) + H_2O \leftrightarrow \text{DeoxyHb- - - } H_2O \quad (15)$$

$$\text{DeoxyHb- - - } H_2O + O_2 \leftrightarrow \text{MetHb } (Fe^{+3}) + O^{-}_{2}\circ \quad (16)$$

4.3 Autoxidation of hemoglobin in meat

The oxygen affinity of hemoglobin varies with globin structure that related to differences in primary structure (Perry and Tuft, 1998). In addition the oxygen affinity is modulated by allosteric interactions. Binding of allosteric effectors such as protons, chloride, and organic phosphates (adenosine triphosphate, ATP) to specific binding sites on the hemoglobin lowers the affinity of the heme groups for oxygen. The heterotropic effectors bind preferentially to hemoglobin in the deoxygenated form, which they stabilize by additional bonds (Perutz, 1990).

Proton binding at distal histidine can produce a substantial decrease of the O_2-affinity, a phenomenon known as the Bohr effect, but the largest decrease is produced by a mechanism that occurs at low pH values, named the Root effect. Then the increasing of DeoxyHb content at post mortem pH values can be explained by the Bohr effect, which is defined as a decrease in oxygenation of hemoglobins with increasing H^+ concentration between pH 7.4 and 6.5 (Stryer, 1988). A further decrease in hemoglobin oxygenation below pH 6.5 is termed the Root effect (Manning *et al.*, 1998). Concerning the Root effect, a mechanism proposed by Mylvaganam *et al.* (1996) has been criticized by Mazzarella *et al.* (1999), suggesting that several combinations of structural characteristics could lead to explain the drastic decrease of O_2-affinity (Pelster and Decker, 2004). Miller *et al.* (1998) found that chicken hemoglobins are mostly deoxygenated form in breast muscle (pH 5.6–5.8). The presence of DeoxyHb has been shown to accelerate MetHb formation compared to fully oxygenated hemoglobin (Shikama, 2006). At post mortem pH values, trout hemoglobin was largely deoxygenated when compared to bovine hemoglobin, while chicken hemoglobin had an intermediate level of DeoxyHb content. Thus the oxygenation depends on the sensitivity to Bohr effect of hemoglobin (Richards and Hultin, 2000). Hemoglobin from trout is largely deoxygenated at pH 6.3 due to a strong Bohr. Harrington *et al.* (1973) reported that trout hemoglobin at pH 7.25 has the higher oxygenation than lower pH values. Moreover, trout

hemoglobin possesses anodic and cathodic types of hemoglobin. The anodic hemoglobins have low oxygen affinity at pH 6.3 whereas cathodic hemoglobins bind oxygen strongly independent of pH (Harrington *et al.*, 1973). Anodic hemoglobins were found to promote lipid oxidation more rapidly than cathodic hemoglobins (Richards *et al.*, 2002). The necessary for trout to have different content of anodic and cathodic hemoglobins is attributed to the variety of environmental oxygen pressures that these fish inhabit (Zolese *et al.*, 1999).

In the presence of ATP, the hemoglobin structure was stabilized in a conformational state similar to the deoxygenated state (Peres *et al.*, 2004). This phenomenon may be partially explained by two amino acid replacements in the β-chains (glutamine to threonine and valine), which result in the loss of two negative charges at the $\alpha_1\beta_2$ interface and favors the dissociation into dimers (Bonafe *et al.,* 1999). The major hemoglobin from *Brycon cephalus* presents a fast partial deoxygenation even in the presence of oxygen that is induced by phosphate binding at pH below 7.0 (Bonilla-Rodrigue and Poy, 2004).

At times of intense activity, frightening, it would increase the amount of oxygen available to tissues and then acidification of the muscle is occurred (Bonafe *et al.*, 1999a). Riggs (1998) stated that a tetramer-tetramer hemoglobin association in birds causes a decrease in oxygen affinity. Thus, the decline in pH occurring in post-mortem stage may establish conditions that tetramers of hemoglobin to associate and thereby increase the content of DeoxyHb in the muscle. In addition, MetHb is at least 60 times more likely to release its heme group compared to OxyHb and DeoxyHb (Hargrove *et al.*, 1996b). An explanation for DeoxyHb-accelerated MetHb formation involves access of oxidants to the heme crevice. H_2O_2 can oxidize DeoxyHb around 100 times faster than OxyHb, which leads to formation of MetHb (Shikama, 2006b). OxyHb is more compact than DeoxyHb which may hind access of H_2O_2 to the oxygenated molecule (Stryer, 1988). In the absence of H_2O_2, it has been proposed that MetHb and $O°_2^-$ formation occurs in tissues due to the oxidation of DeoxyHb by free O_2 (Brown, 1962).

5. Roles of hemoglobin on lipid oxidation

5.1 General Aspects

An important consideration to the role of hemoglobin on catalysis of lipid oxidation in muscle foods is the residual hemoglobin content in muscle tissue. Kranen *et al*. (1999) reported that only detectable heme pigment in breast muscle from bled broilers was hemoglobin while myoglobin was undetectable. In dark muscle of the broilers, 86% of the total heme protein was hemoglobin on a weight basis. However, at least 30% of the total heme protein in beef was hemoglobin (Fox, 1966). However, in sockeye salmon, there was no significant difference in hemoglobin levels estimated in whole muscle from bled and unbled fish. It implied that some case bleeding just can remove the little amount of hemoglobin from the muscle (Porter *et al.*, 1992). An important reaction related to the ability of hemoglobin to stimulate lipid oxidation is hemoglobin autoxidation. This occurs when oxygen was released from OxyHb (such as in case of pH drop or in the present of ATP etc.) to form ferric (+3) MetHb and the superoxide anion radical ($O°_2^-$). $O°_2^-$ would readily be converted to H_2O_2, and the reaction of MetHb with H_2O_2 caused the formation of a ferryl protein radical, an initiator of lipid oxidation (Kanner and Harel, 1985).

Iron catalysis related to the lipid oxidation, since iron compounds were mostly involved in oxygen transport, oxygen storage, and oxygen activation in the form of heme pigments , including myoglobin and hemoglobin (Skibsted *et al.*, 1998). The participating of heme-iron on oxidative deterioration in meat involved both one- and two-electron transfer processes. The catalytic mechanisms of iron catalysis were different from the lipoxygenases catalysis of lipid oxidation with the different environment for iron. Further, when a critical level of peroxides was present, iron could be released from hemin to participate in lipid oxidation processes (Puppo and Halliwell, 1988a). There was evidence to support acceleration of lipid oxidation by DeoxyHb, although its pathway was not clear (Binotti *et al.*, 1971a). The mackerel hemoglobins were found to promote lipid oxidation in washed cod more rapidly than trout hemoglobins (Richards and Hultin, 2000b).

5.2 Pro-oxidative iron species

The ability of iron to alternate between oxidation states makes its reactive in oxidation reactions where electrons are transferred to or from organic compounds. In acidic aqueous solution, the simplest form of iron is found as $Fe(H_2O)_6^{2+}$ or $Fe(H_2O)_6^{3+}$, each coordinating six water molecules. Most salts of iron(II) are fairly soluble in acidic and neutral solution, but $Fe(H_2O)_6^{2+}$ is readily oxidized by O_2 to $Fe(H_2O)_6^{3+}$, which will deprotonise and form soluble hydroxy complexes or precipitate as hydroxy-polymers (Silva *et al.*, 1990; Silver, 1993). The most stable form of iron at physiological oxygen concentrations is complexes of ferric form (Fe^{3+}). Oxidation of organic compounds in the presence of iron and hydrogen peroxide is usually termed "Fenton chemistry". The reactive oxygen species, the hydroxyl radical ($^{\bullet}OH$), the superoxide anion ($O_2^{\bullet-}$) and its conjugate acid ($HO_2^{\bullet}$) are involved in Fenton chemistry, is widely referred to as equation 17 and is regarded as a common source of hydroxyl radicals.

$$Fe^{2+}+H_2O_2 \longrightarrow Fe^{3+}+HO^{\bullet}+OH^{-} \qquad (17)$$

$OH^{\bullet}$ fairly explains the ability of mixtures of iron species and H_2O_2 to initiate oxidation in various systems. It has been proposed that the reactive product of the reaction of equation 18 is not a hydroxyl radical, but an iron-oxo (FeO^{2+}) or ferryl species (Fe(IV)=O), with iron in the oxidation state +4 (Serdar *et al.*, 2000; Dubuisson *et al.*, 2001; Dunford, 2002). A two-electron transfer and not a one-electron transfer thus results from the reaction between Fe^{2+} and H_2O_2:

$$Fe^{2+}+H_2O_2 \longrightarrow FeO^{2+}+H_2O \qquad (18)$$

It is probable that both hydroxyl radicals and ferryl iron species are formed in relative amount depending on the actual system (Parikka *et al.*, 2006).

5.3 Cleavage of peroxides

Besides reductive cleavage of peroxides by iron (II)-species (equation 19 and 20) peroxides may also be cleaved by iron (III) species:

$$Fe^{3+}+H_2O_2 \rightarrow FeO^{2+}+HO^{\bullet}+H^{+} \quad (19)$$

or in analogy with equation 20, as a two-electron transfer reaction with an iron–oxo species (an iron(IV)=O or perferryl species) as reaction product:

$$Fe^{3+}+H_2O_2 \rightarrow FeO^{3+}+H_2O \quad (20)$$

The presence of hydrogen peroxide is accordingly critical for biological systems, and the mechanism and extent of H_2O_2-formation has been extensively studied (Poppo and Halliwell, 1988). $O_2^{\bullet-}$ is readily formed in autoxidation of various compounds such as ascorbate (Wagner *et al.*, 1996) or oxymyoglobin (Brantley *et al.*, 1993). Not only hydrogen peroxide but also organic peroxides such as the lipid hydroperoxides (LOOH) react with iron species. The lipid alkoxyl radical, $LO^{\bullet}$, is reactive and able to abstract a hydrogen atom from another lipid hydroperoxide accelerating lipid oxidation through additional propagation cycles. The reactions of equation 21-24 depend on the existence of preformed LOOH, and this route of oxidation has accordingly been termed "LOOH-dependent oxidation" (Minotti, 1993).

$$Fe^{2+}+LOOH \rightarrow Fe^{3+}+LO^{\bullet}+OH^{-} \quad (21)$$

$$Fe^{2+}+LOOH \rightarrow FeO^{2+}+LOH \quad (22)$$

$$Fe^{3+}+LOOH \rightarrow FeO^{2+}+LO^{\bullet}+H^{+} \quad (23)$$

$$Fe^{3+}+LOOH \rightarrow FeO^{3+}+LOH \quad (24)$$

5.4 Routes of hemoglobin (and hemes) association in lipid oxidation

The cleavage of peroxides by iron species may be oxidative or reductive. The iron becomes catalytic active with the different iron species with different efficiency. The suggested catalytic mechanisms for different iron species was presented in Table 1. Iron catalysis mechanisms for oxidation systematized as one- or two-electron transfers and with Fe (II) or Fe(III) as reductant. Redox states involved in the ***1st Rx*** of the mechanisms are shown in Table 1.

Table 1 Mechanisms of iron catalysis

Route	Mechanism (*n* ë transfers)	re. / ox.	Descriptions	References
1.	Non-heme-iron catalysis (1 and 2 ë) ox_1 — non-heme Fe^{2+} — ox_2 ***1st Rx*** ; ***2nd Rx*** red_1 — non-heme Fe^{3+} — red_2	Fe^{2+} ⇅ Fe^{3+}	The formation of HO° radicals through Fenton chemistry (ox_1= H_2O_2) to oxidize ferrous to ferric, and the second reaction, the oxidation of H_2O_2 by ferric.	(Reed, 1982; Silver, 1993; Wagner *et al.*, 1996)
2.	Heme-iron catalysis : based on the studies mainly myoglobin, hemoglobin and non-protein bound heme-iron			
2.1	Fenton-like mechanism (1ë) LOOH — heme Fe^{2+} — $LOO° + H^+$ ***1st Rx*** ; ***2nd Rx*** $LO° + HO^-$ — heme Fe^{3+} — LOOH	Fe^{2+} ⇅ Fe^{3+}	Is a pro-oxidative activity of heme- iron, a lipid alkoxy radical is formed in the ***1st Rx*** and reduction of heme-ferric by H_2O_2.	(Binotti *et al.*, 1971a; Puppo and Halliwell, 1988a; Schaich, 1992; Kanner and Lapidot, 2001)
2.2	Iron (III)/Iron (IV) mechanism (1ë) LOOH — heme Fe^{3+} — $LOO° + H_2O$ ***1st Rx*** ; ***2nd Rx*** $LO° + H^+$ — heme $Fe^{4+}=O$ — $LOOH + H^+$	Fe^{3+} ⇅ Fe^{4+}	In the***1st Rx***, heme ferryl was formed 1ë plus an oxygen atom is transferred from a LOOH and ***2nd Rx***, heme ferryl will oxidative cleavage of LOOH to LOO°.	(Baron *et al.*, 1997; Everse and Hsia, 1997; Kanner and Lapidot, 2001)
2.3	Pseudoperoxidase mechanism (2 ë) LOOH — heme Fe^{2+} — $LOO° + H^+$ ***1st Rx*** ; ***2nd Rx*** $LO° + HO^-$ — heme Fe^{3+} — LOOH proteins, lipids — ***3rd Rx*** LOOH — heme $Fe^{4+}=O$ — H_2O	Fe^{2+} → Fe^{3+} ⇅ Fe^{4+}	A 2 ë transfer from heme ferric takes place, heme ferric and subsequently heme ferric is regenerated by receiving an electron from each of two electron donors: e.g. lipid and protein molecules (or peroxides), the catalytic cycle may both initiate and propagate oxidation.	(Chance *et al.*, 1986; Kröger-Ohlsen *et al.*, 2002)
2.4	Synproportionation mechanism or auto-reduction LOOH — heme Fe^{2+} — 2 heme Fe^{3+} ***1st Rx*** LOH — heme $Fe^{4+}=O$ — heme $Fe^{2+} + H^+$	Fe^{2+} → Fe^{4+} → Fe^{2+} → $2Fe^{3+}$	Heme-ferrous species can be oxidized by 2ë transfer when mixed with peroxides to form ferryl state. But found the formation of heme-ferric in the reaction. Because heme-ferryl reacted with heme-ferrous in a so-called synproportionation reaction.	(Whitburn, 1985; Yusa and Shikama, 1987; Whitburn and Hoffman, 1987 ; Giuliv and Davies, 1990)

Abbreviations: ***1st Rx, 2nd Rx, 3rd Rx***, they mean the first, second, and third reaction; re. is reduction; ox is oxidation; Fe^{2+}, Fe^{3+}, Fe^{4+} represent ferrous, ferric, and ferryl heme-iron, respectively; ë is electron; LOO°, LO°, °OH, °OOH, and LOOH represent lipid peroxyl, lipid alkoxyl, hydroxyl, peroxyl radicals and lipid hydroperoxides, respectively.

Sources: Modified from Carlsen *et al.* (2005) and references

5.4.1 Non-heme-iron catalysis

The ligands, which bind to non heme-iron under the actual conditions, as shown in route 1 in Table 1, are important for the redox-activity and ability to act as a catalyst (Reed, 1982; Silver, 1993). As an example, the formation of hydroxyl radicals through Fenton chemistry is likely to be catalyzed by some non-heme-iron species. The ***1st Rx*** was the first reaction of route 1 in Table 1 (where H_2O_2 (ox_1) is reduced to $^{\bullet}OH$ and ^{-}OH), but as the second reaction (***2nd Rx***) in Table 1, the oxidation of H_2O_2 by non-heme Fe^{3+} is in most cases too slow to allow efficient catalysis. The mechanism of non-heme-iron catalysis of Table 1 is only valid for one-electron transfers. If two-electron transfer becomes significant, the mechanism indeed becomes more complex (Dunford, 2002; Parikka *et al.*, 2006).

5.4.2 Heme – iron catalysis

Different mechanisms have been suggested for the catalysis of oxidation by heme-iron, based on studies with mainly hemoglobin and myoglobin. Non-protein bound heme-iron may be released from hemoglobin or myoglobin under certain conditions, and heme (ferroproto-porphyrin) is in solution mainly found as hematin (ferriprotoporphyrin hydroxide). Heme, hematin and hemin are normally used interchangeably to describe the existence of non-protein bound heme-iron (or "free heme-iron"). Hemin is ferriprotoporphyrin chloride, which readily converts to hematin in aqueous solution, and accordingly the term hematin should be used for non-protein bound heme-iron.

5.4.2.1 Fenton-like mechanism (Heme-Fe^{2+}/Heme-Fe^{3+})

A Fenton-like mechanism of catalysis is suggested to account for the pro-oxidative activity of heme-iron species (Puppo and Halliwell, 1988b; Schaich, 1992; Kanner and Lapidot, 2001). A hydroxyl radical, or in the case of LOOH reduction is $LO^{\bullet}$, which formed in the ***1st Rx*** of the catalytic cycle in route 2.1 of Table 1. Reduction of heme-Fe^{3+} by H_2O_2 is sometimes proposed as the ***2nd Rx*** of the catalytic cycle, although it is slower than the ***1st Rx***. The heme-iron catalysis through the Fenton-like mechanism is also influenced by the high oxygen affinity of hemoglobin and myoglobin, which generally results in rapid oxygenation of $MbFe^{2+}$ and $HbFe^{2+}$ at normal physiological oxygen concentrations (Binotti *et al.*, 1971a). Deoxyheme- Fe^{2+} proteins available for Fenton-like chemistry is thus dependent on the oxygen dissociation rate constant of the heme- Fe^{2+}--O_2 protein at the actual oxygen pressure.

5.4.2.2 Heme-Fe^{3+}/heme-Fe^{4+} mechanism

Oxidation of heme-Fe^{3+} to iron species of higher oxidation states (hypervalent iron) is well established to occur in reactions with peroxides (King and Winfield, 1963; Everett *et al.*, 1996; Akkara *et al.*, 2000) and the visible absorption spectra of hypervalent forms of myoglobin and hemoglobin are characteristic and easily distinguished from absorption spectra of the Fe^{2+}- or Fe^{3+}-oxidation states of the heme proteins (King and Winfield, 1963; Everett *et al.*, 1996; Akkara *et al.*, 2000). In the mechanism shown in route 2.2 of Table 1, one-electron transfer takes place between heme-Fe^{3+} and LOOH. In the ***1st Rx*** of the mechanism one electron plus an oxygen atom is transferred from a peroxide to the heme group. The last electron necessary for the iron-oxygen bond is formally from Fe^{3+}, which is oxidized to Fe^{4+} (Everse and Hsia, 1997). Regeneration of heme-Fe^{3+} in the ***2nd Rx*** of the mechanism is possible as the heme-Fe^{4+}=O species is strongly oxidizing. And oxidative cleavage of LOOH to $LOO^{\bullet}$ by $MbFe^{4+}$=O is favorable in meat.

5.4.2.3 Pseudoperoxidase mechanism (heme-Fe^{2+}/heme-Fe^{4+})

In the pseudoperoxidase mechanism, a two-electron transfer from heme-Fe^{3+} takes place, and subsequently heme-Fe^{3+} is regenerated by receiving an electron from each of two electron donors. As these donors may be lipids or proteins (or peroxides hereof), the catalytic cycle may both initiate and propagate oxidation (Kröger-Ohlsen *et al.*, 2002). In the two-electron transfer reaction (***1st Rx*** in route 2.3 of Table 1) an oxygen atom from the peroxide binds to the heme-iron and the two electrons for the iron–oxygen bond are formally originating from the heme-iron species (Everse and Hsia, 1997). One electron formally comes from Fe^{3+}, which is oxidized to Fe^{4+}, and the other comes from the porphyrin ring, which is oxidized to a porphyrin radical cation (Kanner and Harel, 1985; Akkara *et al.*, 2000). For myoglobin and hemoglobin, this radical cation immediately oxidizes an amino acid residue of the surrounding peptide chain, leaving the perferryl species as a protein radical with iron in the oxidation state +4 (Everse and Hsia, 1997). Most studies on pseudoperoxidase activity have been performed with myoglobin, and even though HbFe(III) is also reported to react with H_2O_2 and oxidize to perferryl and ferryl states (Everett *et al.*, 1996), only incomplete information exists for the rate of activation of $HbFe^{3+}$ and for the reactivity of hypervalent Hb states (Everse and Hsia, 1997).

5.2.2.4 Synproportionation mechanism (heme-Fe^{2+}/heme-Fe^{4+})

Heme-Fe^{2+} species can also be oxidized by two-electron transfer when mixed with peroxides, as indicated by the detection of $MbFe^{4+}$=O in reaction mixtures of $MbFe^{2+}$ and H_2O_2 (Yusa and Shikama, 1987). The oxygenated forms of the heme pigments have also been reported to form the ferryl states upon reaction with H_2O_2 (Whitburn and Hoffman, 1987 ; Giuliv and Davies, 1990), but results from the study on myoglobin by Yusa and Shikama (1987) indicate that $MbFe^{2+}O_2$ seems to be deoxygenated and affect its reaction with H_2O_2. $MbFe^{2+}$/$HbFe^{2+}$ must be regenerated from $MbFe^{4+}$=O or $HbFe^{4+}$=O, respectively, if a catalytic reaction cycle should be functioning (i.e. a catalytic heme-Fe^{2+}/heme-Fe^{4+}=O mechanism). Such regeneration, the ferryl states preferentially reduce to ferric states in one-electron transfer reactions (route 2 in table 1). Actually, mixtures of $MbFe^{2+}$/$MbFe^{2+}O_2$ and H_2O_2 are rapidly converted into $MbFe^{3+}$, because $MbFe^{4+}$=O reacts with $MbFe^{2+}O_2$/$MbFe^{2+}$ in a so-called “synproportionation reaction” as showed in (25) (Yusa and Shikama, 1987; Whitburn and Hoffman, 1987).

$$MbFe^{4+} = O + MbFe^{2+} + 2H^{+} \longrightarrow 2MbFe^{3+} + H_2O \qquad (25)$$

A synproportionation reaction has also been shown to take place for hemoglobin (Giuliv and Davies, 1990), and generally synproportionation accelerates the rate of reduction of the heme-Fe^{4+}= O species relative to autoreduction (Whitburn and Hoffman, 1987 ; Giuliv and Davies, 1990).

6. Antioxidants

6.1 General aspects

Numerous studies have indicated that lipid oxidation may be controlled or at least minimized through the use of antioxidants, substances that protect other chemicals from oxidative damaging with free radicals and other oxygen species. During this reaction, the antioxidant sacrifices itself by becoming oxidized. However, antioxidant supply is not unlimited as one antioxidant molecule can only reacts with a single from radicals. In the living cells process several protection mechanisms directed against lipid oxidation products. Enzymatic antioxidants include: glutathione peroxidases, which reduces hydroperoxides in the cellular membranes to the

corresponding hydroxy compounds or water; superoxide dismutase (SOD), which catalyses the conversion of $°O_2^-$ to H_2O_2 and H_2O; catalase, which then converts H_2O_2 to H_2O and O_2, they are sources of endogenous antioxidant in living cells (Bulger and Helton, 1998). And also, non-enzymatic antioxidants include the lipid soluble vitamins such as alpha-tocopherol, riboflavin or pro-vitamin A (beta-carotene), and water soluble vitamins such as ascorbic acid and glutathione.

Alpha-tocopherol has been described as the major chain-breaking antioxidants in the living cells (Pecker, 1992). Its lipid soluble property functions from a long aliphatic side chain which gives the lipid solubility. Thus the alpha-tocopherol can located within cellular membranes, where it interrupts lipid peroxidation(Kagan *et al.*, 1990; Azzi *et al.*, 1993). Citric acid, ethylene diamine tetra acetic acid (EDTA), phosphoric acid, and ascorbic acid exhibited metal chelating activity thus these compound could inhibit lipid oxidation (Buckley *et al.*, 1995; El-Alim *et al.*, 1999; McCarthy *et al.*, 2001a; Sánchez-Escalante *et al.*, 2003; Ahn and Nam, 2004). The purpose of these compounds is to take the free radicals out of the reaction and to prevent them from being involved in the propagation step. The antioxidant molecule (ArOH) is converted to a free radical (ArO°) which does not aid in the propagation reactions (equation 26 and 27). In the foodstuffs, BHA or BHT was used more frequently than natural antioxidants such as the tocopherols.

Chain breaking antioxidants

$$ROO° + ArOH \rightarrow ROOR + ArO° \quad (26)$$

$$ROO° + ArO \rightarrow \text{non-radical products} \quad (27)$$

6.2 Natural antioxidants

Synthetic antioxidants have long been used, but their side effects can cause a suspected carcinogenic potential (Chen *et al.*, 1992) and the general rejection of synthetic food additives by consumers. Several studies have documented the effectiveness of antioxidative components in herbal plants such as flavonoids, phenolic or polyphenolic and related compounds for the prevention of lipid oxidation in meat and meat products (Cuvelier *et al.*, 1996; Botsoglou *et al.*, 2002; Ahn and Nam, 2004; Aleson-Carbonell *et al.*, 2005). The antioxidant activity of phenolic compounds in herbs and spices is mainly due to their reduction properties and chemical structures,

which can act as reducing agents, free radical scavengers, Fe^{2+}- chelators or quenchers of the formation of singlet oxygen (Zheng and Wang, 2001; Pizzale *et al.*, 2002).

In recent years many researcher has been focused on extracts from herbs and spices, which have been used traditionally for centuries to improve the sensory characteristics and to extend the shelf-life of foods. The antioxidant properties of herbs and spices are related to their phenolic contents. Thus, their antioxidant action is similar to synthetic phenolic antioxidants. The culinary herbs and spices such as lemon grass, ginger, galangal and holy basil are widely used in Thai cooking (Cousminer and Hartman, 1996; Wang *et al.*, 1996).

6.2.1 Galangal

Galangal or greater galangal (*Alpinia galanga*), a rhizome closely related to the ginger family, is native to Southern China and Thailand , has been one of the most important ingredients in Thai curry paste and is commonly used as flavouring (Wang *et al.*, 1996). Galangal rhizome is primarily used as a flavoring especially in the preparation of fresh. Several researchers had reported that galangal extracts showed antioxidant activity in many model systems (Wang *et al.*, 1997; Cheah and Abu-Hasim, 2000b; Javanmardi *et al.*, 2003). Two phenolic compounds such as *p*-hydroxycinnamaldehyde and di-(*p*-hydroxy-*cis*-styryl) methane were isolated from the chloroform extract of the rhizomes of *A. galanga* (Barik *et al.*, 1987). The potent antioxidant activity of curcuminoids isolated from *Alpinia galangal* was reported that in the essential oil from rhizomes of greater galangal comprised of 1,8-cineole, beta-pinene, alpha-terpineol, fenchyl acetate, alpha-pinene, camphene, guaiol, camphor and beta-elemene (Jitoe *et al.*, 1992; Narasimhamurthy and Raina, 1999). Cheah and Abu Hasim (2000) investigated the antioxidative effect of galangal in raw and cooked minced beef during storage at 4 °C. It was found that thiobarbituric acid (TBA) values of raw and cooked samples containing galangal extract at 5 and 10 % (w/w) were significantly ($p<0.05$) lower than those of the controls. And in raw beef, galangal extract at 10 % (w/w) was as effective as alpha-tocopherol at 0.10 % (w/w) and butylated hydroxytoluene (BHT) at 0.02 % (w/w) in minimising lipid oxidation.

6.3.2 Lemon grass

Lemon grass is a Thai food ingredient, an aromatic tropical grass containing a lemony scent similar to lemon balm. Lemon grass oil consists of 75-85 % of citral (neral and geranial), methyl heptenone, citronellal, geraniol, limonene, dipentene, camphor, seven mono- and

eight sesquiterpene hydrocarbons and traces of methyl eugonol (Windholz *et al.*, 1983). The extracts from lemon grass showed antioxidative and antimycotic effect on butter cakes (Lean and Mohamed, 1999). Additionally, Cheel *et al*. (2005) reviewed that the components of polar extract of lemon grass are isoorientin, homoorientin, isoscoparin, swertiajaponin, 7-*O*-methylisoorientin, isoorientin, 2' '-*O*-rhamnoside, 7-*O*-methyl-6-β-D-glucopyranosylluteolin, orientin, chlorogenic acid, and caffeic acid (Figure 6)(Cheel *et al.*, 2005).

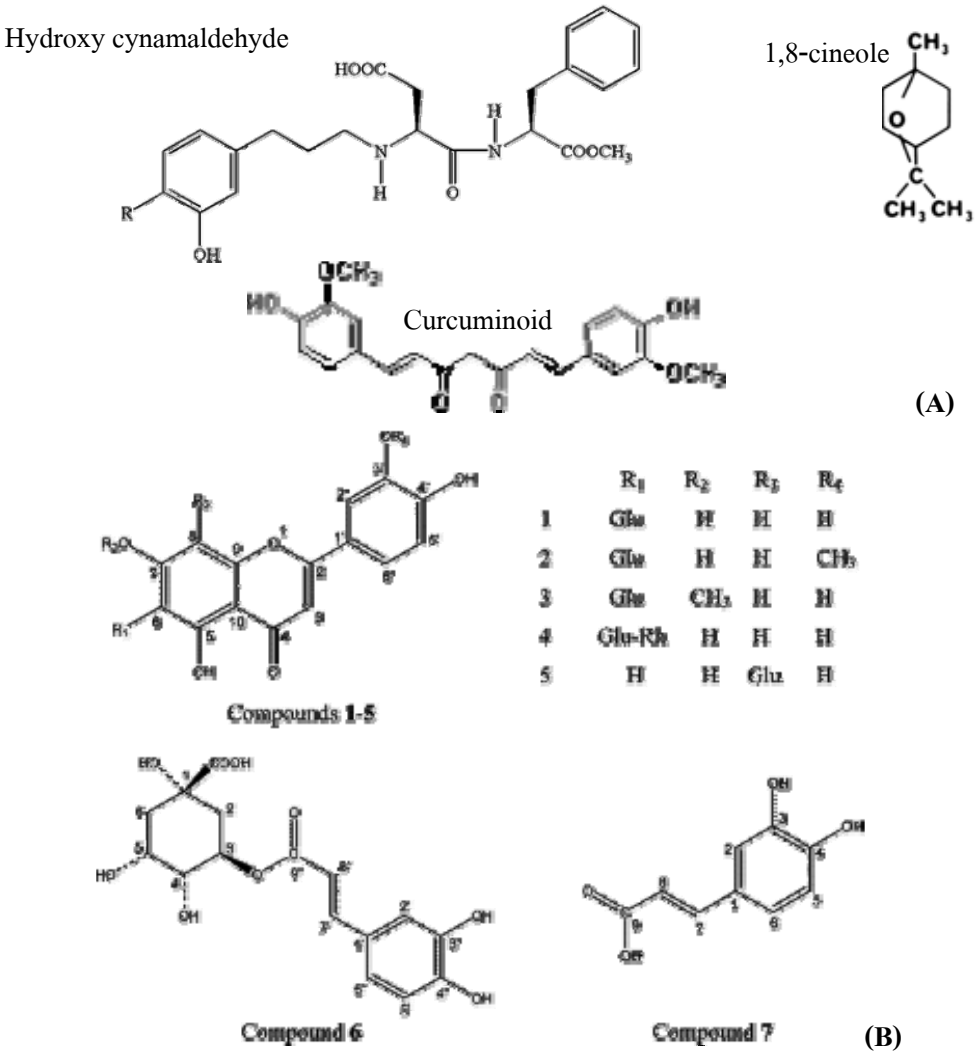


Figure 6 Some polyphenolic compounds found in galangal (A) and lemon grass (B), Isoorientin, homoorientin (**1**), isoscoparin (**2**), swertiajaponin, 7-*O*-methyl-6-β-D-gluco pyranosylluteolin, 7-*O*-methylisoorientin (**3**), isoorientin, 2' '-*O*-rhamnoside (**4**), orientin (**5**), chlorogenic acid (**6**) and caffeic acid (**7**). Glu abbreviated from a glucose unit.

Sources: Cheel *et al.* (2005); Kawahara *et al.(*2004)

7. Natural antioxidants on lipid oxidation mediated by heme proteins

The ability of phenolic components to inhibit lipid oxidation in muscle food systems may be attributed to free radical scavenging activity (Robak and Gryglewski, 1988; Jovanovic *et al.*, 1996), metal chelating capacity (Sergent *et al.*, 1993; Brown *et al.*, 1998), and the deactivation of hypervalent ferryl heme pigments known to initiate lipid oxidation (Hu and Skibsted, 2002b). To assess antioxidant effectiveness in muscle food, a washed fish muscle added hemoglobin was elaborated (Pelster and Decker, 2004). Lee *et al.* (2006) reported that proanthocyanin-enriched fraction of cranberry extract was the potent efficient scavenger of DPPH radical compared to other fractions but it was weakly inhibitory to hemoglobin-mediated lipid oxidation compared to other fractions. However, the ability of components to scavenge DPPH radicals does not adequately predict their ability to inhibit Hb-mediated lipid oxidation in washed cod muscle (Lee *et al.*, 2006).

Moreover, the quantity of phenolate groups can relate poorly to the degree of inhibition of lipid oxidation processes. For example catechin, epicatechin and quercetin each contain 5 phenolate groups per molecule, but their ability to inhibit Fe^{2+}-mediated lipid oxidation in liposomes varied widely (Liao and Yin, 2000). Consistent with the partitioning, catechin more effectively inhibited Fe^{2+}-mediated lipid oxidation compared to quercetin (2.2-fold difference) and epicatechin (1.2-fold) (Lee et al., 2000). Therefore, it was proposed that the physical location of the inhibitory components was more important in dictating inhibitory efficacy than the number of phenolate groups that were present. The binding affinity of phenolics to insoluble muscle components (e.g., myofibrillar proteins and membrane phospholipids) could influence the ability of phenolics to inhibit lipid oxidation processes. A previous research reported that the proanthocyanidin-enriched fractions of cranberry most effectively increased the lag time of Cu^{2+}-induced low density lipoprotein (LDL) oxidation compared to other fraction (Porter *et al.*, 1992). Apparently the proanthocyanidins remained associated with the LDL during purification whereas the other fractions were washed away. Lee *et al.* (2006) found that flavonols richer fraction was more effective than hydroxycinnamic acid fraction in inhibiting TBARS formation in washed cod when a washing step was employed. The higher binding affinity for insoluble components in washed cod muscle was a better inhibitor of hemoglobin-mediated lipid oxidation (Lee *et al.,* 2006).

Goupy *et al.* (2007) proposed a possible mechanism that the quercertin and their degraded products (Figure 7A) displayede as a synergistic to tocopherol in deactivation of oxo-ferryl heme. This complex interplay between quercetin, α-tocopherol, and their oxidation products could take place close to the heme cavity where the quercetin reacts with the iron-oxo center. Indeed, although α-tocopherol apparently inerted toward the iron-oxo center, it efficiently protected the heme from degradation during the met-heme induced peroxidation of linoleic acid (Vulcain *et al.*, 2005; Goupy *et al.*, 2007). Assuming a mostly heterolytic cleavage of the peroxo bond during the initiation step, this protection could operate via a fast electron transfer from α-tocopherol to the porphyrin radical cation (Figure 7 B). Thereafter, the porphyrin nucleus thus generated could in turn to act as an electron donor toward the radicals derived from quercetin or its oxidation products (Figure 7C). Hence, a porphyrin-mediated regeneration of quercetin and/or some of its oxidation products by α-tocopherol could provide the basis for the synergism observed between these antioxidants.

The ability of flavonols to orient in the membrane bilayer had been shown (Van den Broek *et al.*, 2001). Having the antioxidant molecule located in the lipophilic membrane would be especially useful in the scavenging of lipophilic hemin radicals and alkoxyl radicals that formed during the reaction of released heme with preformed lipid peroxides (Tappel, 1955). The ability of the flavonol fractions to effectively inhibit lipid oxidation in washed cod might be due to the ability of flavonols to modulate the biomembrane fluidity in such a way that limits the diffusion of free radicals (Arora *et al.*, 2000; Caturla *et al.*, 2003). Hu and skibsted (2002) reported that epigallo catechin gallate in green tea extract and green tea polyphenol extract had a potential to reduce the hypervalent heme pigments, deactivation of ferryl heme. Moreover, green tea polyphenols were effectively inhibition of the lipoxygenase activity in mackerel muscle (Banerjee, 2006).

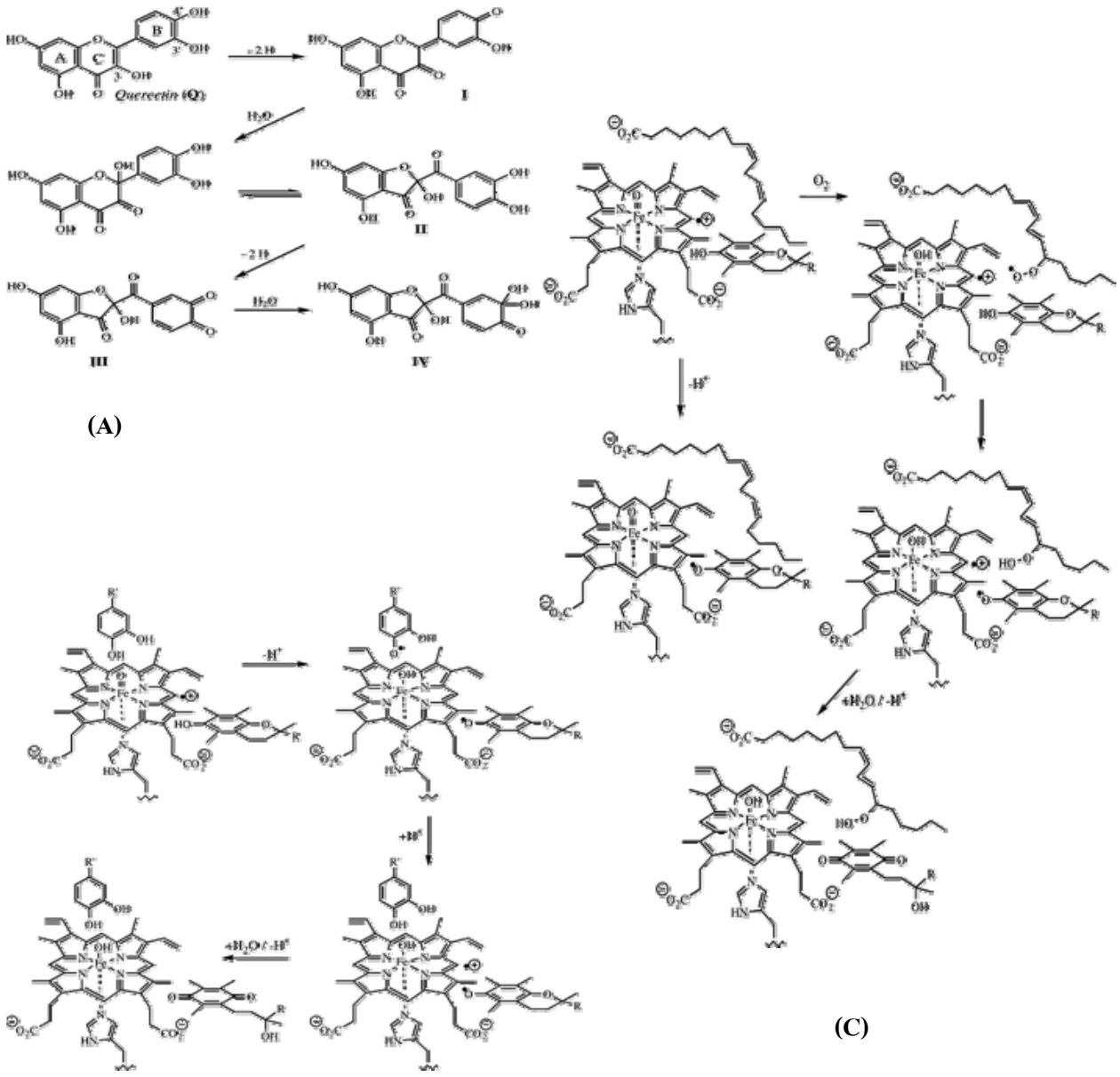


Figure 7 Model of polyphenol on heme porphyrins oxidation: **(A)**, the oxidation of quercetin by Fenton like reaction, **(B)** the inhibition of peroxidation by α-tocopherol and **(C)** the inhibition of initiation by quercetin.

Source: Vulcain *et al*. (2005) and Goupy *et al*. (2007)

Research hypothesis

It was hypothesized that deoxygenation of sea bass hemoglobin would stimulate by exposure to acidic pH and/or to ATP. The consequences accumulation of DeoxyHb would be a great pro-oxidant taking part for lipid oxidation was expected to occur. However the peroxidative activity of DeoxyHb may associate with other fish physiological conditions during postmortem process that related to amount of endogenous pro- and anti-oxidant components. For instance, the filleting just-after death or pre-rigor fish was expected to facilitate spreading of liquid blood on fish surface which is rich of nucleotide residue. Thus, an increase of DeoxyHb having oxidizing activity was expected. It was also hypothesized that the extracts of galangal and lemon grass would possess antioxidative activities. Therefore washing the fish slices chunk with the extract solutions would prolong lipid oxidation stability in fish.

Research objectives

1. To investigate the effect of pH and ATP on deoxygenation of sea bass hemoglobin
2. To evaluate effect of pH and addition of hemoglobin on lipid oxidation in sea bass minces prepared from the fish at different postmortem stages
3. To determine the antioxidant activities of the aqueous and ethanolic extracts of galangal and lemon grass
4. To investigate the effectiveness of galangal and lemon grass extracts as fish washing solution on retardation of lipid oxidation in fish muscle

CHAPTER 2

MATERIALS AND METHODS

1. Materials and instrumentals

1.1. Raw materials

1.1.1. Live sea bass (*Lates calcarifer*), raised in the shore of Yor island, Songkhla Lake, Songkhla province were purchased and used throughout this experiment.

1.1.2. Tongol tuna (*Thunnus tongol*) was obtained from the port of Songkhla.

1.1.3. Galangals (*Alpinia galanga* (L.) Willd.) aged 1.0-1.2 m and lemon grass (*Cymbopogon citratus* (DC.) Staph) shoot sized 0.2-1.5 cm (x.s.) were purchased from Plaza fresh market, Hat Yai.

1.2. Chemicals

2,2-Diphenyl pycryl hydrazyl, 3-amino benzoic acid ethyl ester, ammonium thiocyanate, bovine serum hemoglobin standard, butylated hydroxy anisol, cumene hydroperoxide, EDTA, ferric chloride, Folin-Ciocalteau, gallic acid, hydrazine, iron (II) chloride, potassium thiocyanate, sodium dithionite, sodium heparin, sodium phosphate dibasic, sodium phosphate monobasic, and tetraethoxy propane were purchased from from Sigma Chemical (St. Louis, MO, USA). Ascorbic acid, chloroform, glycerol, hydrochloric acid, methyl alcohol, sodium carbonate, sodium chloride, thiobarbituric acid, and trichloroacetic acid were obtained from Fluka Chemical (Buchs, Switzerland).

1.3. Instrumentals

Table 2 Instruments

Instruments	Model	Company/Country
Blender	Moulinex	France
Centrifuge	Z 323	HERME, USA
Rotary evaporator	N-100	EYELA, Japan
Homogenizer	POLYTRON® PT-MR 2100	KINEMATICA, Switzerland
Optical Emission spectrometer	Optima 4300 DV	Perkin Elmer Instrument, USA
Microplate reader	3550	Bio-Rad, USA
pH meter	350	Mettler, USA
Refrigerate centrifuge	RC-5B	Sorvell, USA
Sonicator	RH 100 H	BANDERLIN *SONOREX*, USA
Spectrometer	V 350	Jasco, Japan
Texture Analyzer	TA-XT2i	Stable Micro System, England

2. Methods

2.1 Compositional analysis of fish muscles

Dorsal meat of sea bass and tongol tuna was removed, skinned and minced before subjected to determine the moisture, protein content (AOAC, 1999), lipid content (Bligh and Dyer, 1959), ash (AOAC, 1999), heme-iron (Hornsey, 1956), non heme-iron content (Carter, 1971; Ahn and Nam, 2004), total iron (Fe) content (Ferrer *et al.*, 2000) and extractable heme protein (Richards *et al.*, 2002).

2.2 Preparation and study of some properties of sea bass hemolysate

2.2.1 Blood collection: Sea bass was anesthetized in amino benzoic acid ethyl ester solution (0.5 g/L). The fish caudal vein was opened then the blood was withdrawn by syringe rinsed with saline sodium heparin solution (30 units/ml) (Richards and Hultin, 2000b). To facilitate the blood flow, pressure was applied into the fish body by hand pressing. The blood was transferred into a plastic tube containing 0.5 ml of the cold heparin solution. The heparinized blood was

centrifuged at 700 × g (Fyhn *et al.*, 1979) to separate the plasma from erythrocyte. The obtained pellet was used for sea bass hemolysate preparation within 2 hr.

2.2.2 Preparation of sea bass hemolysate: The erythrocyte from 2.2.1 was used for sea bass hemolysate preparation. The hemolysate was prepared as the method of Fyhn *et al*. (1979).

2.2.2.1 The concentration of hemoglobin determination was followed the Drabkin's method (Drabkin and Austin, 1935)

2.2.2.2 The determination of sea bass hemoglobin spectrum pattern from 650-350 nm according to the method of (Rifkind, 1972)

The average values of each analysis were obtained from 3 lots.

2.3 Effect of pH and ATP on oxygenation of sea bass hemolysate

2.3.1 Effect of pH: The hemolysate was diluted with 50 mM phosphate buffer at pH 5.50, 5.75, 6.00, 6.25, 6.50, 6.75, 7.00, 7.25, and 7.50 in the microplate to obtain the appropriate concentration of hemoglobin that provided the absorbance in a range of 0.2 to 0.8.

2.3.2 Effect of ATP: The hemolysate was diluted amd mixed with ATP to obtain the final concentration of 0, 0.5, and 1.0 mM. The pH was adjusted to three values where the most form of hemoglobin was in oxy or deoxy-state according to the 2.3.3.

2.3.3 Hemoglobin autoxidation analysis: Samples from 2.3.1 and 2.3.2 were incubated at 15°C in the microplate reader (HERMLE) then the oxygenation properties of hemoglobin at various conditions were monitored as:

1. Absorbance spectrum from 650-350 nm of diluted hemolysate was conducted according to the method of Rifkind (1972)

2. Percent relative oxygenation of the diluted hemolysate was calculated as the method of Richards and Hultin (2000)

The experiments were designed as factorial (pH× concentration of ATP × time) in Complete Randomized Design (CRD). The experiments were conducted at least three lots. The mean values were subjected to analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine significant ($P < 0.05$) differences between treatments. The SPSS version 12 was used to perform the data analysis.

2.4 Effect of pH and fish postmortem stages on pro-oxidant activities of sea bass hemolysate

Sea bass was anesthetized as section 2.2.1 and kept in polystyrene box containing ice in the ratio of fish to ice as 1:3. The fish was withdrawn at three different postmortem stages: 2, 10 and 48 h after death. The differences in firmness of flesh from the dorsal part were used for identification of the rigor mortis stage (Siripongvutikorn, 2004). Then the fish muscle was prepared as the unwashed and washed minces (Richards *et al.*, 2000). The minced was washed twice in distilled water at a 1:3 mince to water ratio (w:w) and stirred for 2 min. Subsequently, the mixture was allowed to stand for 1 min before dewatering through cheesecloth. The mince then was mixed with 50 mM sodium phosphate buffer (pH 6.2 or 7.0) at the same ratio and homogenized using POLYTRON® PT-MR 2100 for 1 min. It was allowed to stand for 15 min and finally it was centrifuged at 1500 xg for 20 min at 4°C. Thereafter, the mince was adjusted to pH 6.2 (sharply deoxygenating of hemoglobin) and 7.0 (highest oxygenation of hemoglobin) immediately. Sea bass hemolysate was added into the sample and adjusted the final concentration to 5.8 μmole/kg muscle then 200 ppm of streptomycin sulfate was added to prevent microbial growth. The moisture content of each treatment was controlled as 90 percent. Samples was stored in polystyrene box containing ice and kept in a chilled room at 4 °C. The development of TBARS (Buege and Aust, 1978) and lipid hydroperoxides values (Richards *et al.*, 2002) were determined every 60 min up to 96 h.

The experiment was designed as factorial (pH× rigor stages × washing × hemoglobin addition × time) in CRD. The experiment was conducted at least three lots. The mean values were subjected to analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine significant differences between treatments. The SPSS version 12 was used to perform the data analysis.

2.5 Evaluation of antioxidative properties of galangal and lemon grass extracts

2.5.1 Extraction: Galangals and lemon grass were washed, trimmed and sliced into 1-2 mm thickness, blended and mixed with 35, 50, 75, 95 percent of ethanol or distilled water with a spices: solvent ratio of 1: 3 (w/v) in a Moulenex blender. The suspensions were incubated at 75°C for 90 min (Jantachote, 2006) and thereafter filtered through Whatman papers no.1. The extracts were evaporated with rotary evaporator (EYELA, n-100) and kept in an airtight container at 4°C until use.

2.5.2 Evaluation: The extracts were investigated for their polyphenolic content and antioxidative activities as followed:

2.5.2.1 Content of polyphenolic compounds in the extracts was determined as the method of Zhu *et al.* (2002) and gallic acid was served as a standard polyphenolic compound.

2.5.2.2 DPPH radical scavenging activity was determined as slightly modified method of Xu *et al.* (2004) and Yokozawa *et al.* (1998) and compared the activity with BHA.

2.5.2.3 Chelating activity was determined as the method of Pazos *et al.* (2005) and compared the chelating activity with EDTA.

The experiments were designed as CRD. The experiments were conducted at least three lots. The mean values were subjected to analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine significant ($P < 0.05$) differences between treatments. The SPSS version 12 was used to perform the data analysis.

2.6 Effectiveness of galangal and lemon grass extracts on inhibition of lipid oxidation mediated by sea bass hemolysate

Preliminary study found that at pH 6.2 deoxygenation of sea bass hemoglobin was stimulated and exhibited the rapid rate of lipid oxidation (Buaneow, *et al.,* 2007). Thus, the sea bass washed mince at pH 6.2 with the presence of 5.8 μmole hemoglobin/kg was used as a model system. The sea bass sized 1.8-2.2 kg was knocked in ice-cold water with a fish /ice water ratio of 1:3 (w/w) and transported to the laboratory. The fish was scaled in ice cold water, filleted, and skinned. Only dorsal part of the fish was minced and washed as the method of Richards *et al.* (2000). The spice extracts as mentioned in section 2.5.1 were added into the washed mince to have the final concentration of polyphenolic compound equivalent to 0.02 % w/w gallic acid. The samples were stored in the polystyrene box containing ice and kept in chilled room at 4 °C for 7 days. The development of TBARS was monitored (Buege and Aust, 1978) every 12 h.

The selected extract was tested in the extension potency of sea bass hemoglobin oxygenation. The extract was added to the hemoglobin solution in a ratio of sea bass hemoglobin to extract (equivalent to gallic acid) of 1mole: 1 mole, at pH 6.5. The 15% ethanol was used as control treatment and gallic acid was used to compare with the selected extract activity. Thereafter the mixture was measured the relative oxygenation with in 10 min as the method mentioned in 2.2.2.

The experiment was designed as Factorial (species ×solvents × time) in CRD. The experiment was conducted at least three lots. The mean values were subjected to analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine significant differences between treatments. The SPSS version 12 was used to perform the data analysis.

2.7 Effectiveness of galangal and/or lemon grass on fish slice

Sea bass sized 1.8-2.2 kg fish was purchased from the same place as mentioned above. Fish was knocked in ice-cold water with a fish /ice ratio of 1:3 (w/w). Tongol tuna (sized 1.5-2.0 kg) was placed in crushed ice with fish/ice ratio of 1:3 (w/w). Then, the fishes were washed with tap water and filleted. The paired fillets technique (Richards et al., 1998) was applied to the sampling method. Thereafter the fillets were skinned then the dorsal parts were cut into stripes with a thickness of 0.8-1 cm (~ 5 cm^2/10 g/piece). The slices were divided into two groups; the first group was treated by dipping in chilled (4 °C) distilled water for 10 min as a control. While second group was treated by dipping in the selected extract solution at the final concentration equivalent to of 2 % w/v gallic acid. All samples were soaked in the ratio of 1:2.5 (w/v) of fish: solution then drained for 5 min on a basket at 4 °C (Ibrahim Sallam, 2007). The samples were placed on a basket over an ice bath in the polystyrene box and kept in chilled room at 4 °C. The samples were taken at day 0, 3, 6, 9, 12 and15 to monitor the values as follows:

2.7.1 TBARS development was mentioned as the method of Buege and Aust (1978)

2.7.2 pH : muscle sample was homogenized in distilled water by ratio 1:10 of muscle to water before measuring by pH meter

2.7.3 Color values (L^*, a^*, and b^*- values) were measured from three different points with a Color Hunter Lab (Ibrahim Sallam, 2007).

The experiment was designed as factorial (species ×solvents × time) in CRD. The experiment was conducted at least three lots. The mean values were subjected to analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to determine significant ($P < 0.05$) differences between treatments. The SPSS version 12 was used to perform the data analysis.

CHAPTER 3

RESULTS AND DISCUSSION

1. Chemical composition of tongol tuna and sea bass

The chemical compositions of dorsal flesh of tongol tuna and sea bass are shown in Table 3. Total protein, ash, total iron, heme-iron, non-heme-iron and extractable heme protein content (as hemoglobin equivalent) in both fishes were significant difference ($p<0.05$). However, there are no significant difference ($p<0.05$) in lipid, moisture, and ash content. It was well known that the chemical compositions of fish are varied depending on the species, nutritional of fish, the state of nutrition, water temperature, salinity, reproductive cycles, season, age, size, sexual variations and others (Monsen, 1985; Erkan and Ozden, 2007). These fishes were characterized as the medium fatty fish based on their lipid content around 5% (Johnsen and loyd, 1992). It was reported that the fishes had different profiles of lipid components, the unsaturated fatty acid content in tuna and sea bass were 64.6-71.3 % (Vlige and Body, 1988) and 52.7 % of lipid (Kizza *et al.*, 1991), respectively.

From the result (Table 3) the iron content in tongol tuna was higher than that of sea bass. This might associate with different physiological among these fishes. Erkan and Ozden (2007) found that the iron contents of sea bream, a red flesh fish (225 mg/kg), were significantly higher than that of sea bass, a white muscle fish (2.47 mg/100 g). Tuna, a pelagic fish, need more oxygen supplying for aerobic respiration in swimming, chasing, and other activities. Therefore, it requires more amounts of oxygen carriers, stores, and co-reactor in biochemical reactions. The intraspecific variation in myoglobin in the dark muscle of fish appears to be directly related to the amount of exercise by the animal (Haard, 1992b).

In addition, the result showed that tongol tuna had higher content of heme-iron (70.75% of total iron) than that of sea bass (36.93% of total iron). The significant difference in heme protein content was in accordance with a difference in color values of these fishes. Tuna tongol muscle was red whereas the sea bass muscle was white or light in color. This remarkable difference was corresponded with the heme content.

Table 3 Chemical composition of tuna tongol and sea bass

Constituent	Tongol tuna	Sea bass
Moisture (g/100g)	78.35±0.39[a]	78.37±0.22[a]
Lipid (g/100g)	4.57±0.49[a]	3.87±0.39[a]
Proteins (g total N/100g)	14.79±1.18[a]	17.11±0.10[b]
Ash (g/100g)	1.14±0.01[a]	1.09±0.01[a]
Total iron (Fe) *(mg/100 g)	5.98±0.02[b]	3.27±0.01[a]
Heme-iron ** (mg/100 g)	4.22±0.07[b]	1.21±0.05[a]
Non heme-iron** (mg/100 g)	2.20±0.21[b]	1.20±0.12[a]
Extractable heme protein** (mg/100 g)[†]	480.40±2.20[b]	378.70±1.60[a]

* ICP-OES method, ** Spectrophotometry method, [†]Values from using of bovine hemoglobin as heme protein standard

Mean ± SD from triplicate determinations

The different superscripts in the same row denote the significant differences ($p<0.05$)

Concentration of heme proteins especially myoglobin and hemoglobin as blood residue and environmental conditions of these heme proteins determined muscle color (Ramos *et al.*, 2005). Since tongol tuna contained high amount of heme-iron relative to that of sea bass, the significant amount of non heme-iron in this fish due to deterioration of intact molecule of heme-iron. It is worth mentioning that the tongol tuna was inferior related to that of sea bass. However, the percentage of non heme-iron compared to total iron of tongol tuna and sea bass were 36.5 and 36.7%, respectively. Gomez-Basuari and Regenstein (1992) reported that heme-iron constituted 46-65 % of the total iron in mackerel fillets.

The observation implied the possibility that tongol tuna might have high proportion of other hemoproteins, included myoglobin, cytochromes, and iron containing enzymes. Myoglobin content was lower than hemoglobin content in mackerel light muscle and trout whole muscle (Richards and Hultin, 2001). Hemoglobin made up 65 and 56% of the total heme protein in dark muscle from unbled and bled mackerel, respectively (Richards and Hultin, 2002). For some fish, hemoglobin may constitute as much as 30% of the heme protein in red muscle accounted for most of hemoprotein of white muscle (Haard, 1992a). In yellowfin tuna, *Neothunnus macropterus*,

hemoglobin ranged from 0.12 to 0.58 mg/100 g of light muscle and 0.50 to 3.50 mg/100g of dark muscle. Of the total heme protein, hemoglobin constituted 15 to 30% of light meat and 5 to 19% of dark meat (Haard, 1992a). Brown (1962) analyzed the muscle of yellowfin tuna and found myoglobin ranged from 0.37 to 1.28 mg/100 g in light muscle and 5.3 to 224.0 mg/100 g in dark muscle. This available information suggested that an active fish like tuna with deep-seated dark muscle would likely to be rich in myoglobin relative to that of superficial dark muscle (Love *et al.*, 1977).

Interestingly, the ratio of heme-iron to non heme-iron between these fishes was obviously different. They were 2:1 and 1:1 for tongol tuna and sea bass, respectively. It was suggested that the high value of the ratio was probably related to the deterioration or denaturation of hemoprotein. Based on this assumption, the high proportion of non heme-iron in sea bass pointed out a progressive rate of heme proteins denaturation in sea bass relative tongol tuna resulting in loss of iron from the heme porphyrins (Kristensen and Purslow, 2001). An increase content of non heme-iron was also a consequence of the oxidative cleavage of the porphyrin ring in heme molecule (Schricker *et al.*, 1982; Gómez-Basauri and Regenstein, 1992; Miller *et al.*, 1994; Estevez and Cava, 2004). Since hemoglobin is situated in the intermuscle system, limiting in protecting factors, thus prone to destructure or deteriorate. There was a report mentioning the ease of hemoglobin to loss, release, or disrupt the porphyrin rings (Marks, 1969). On the other hand, myoglobin is retained by the intracellular structure (Livingston and Brown, 1981). It is likely to persist against the environmental changes. In contrast, sea bass, a white flesh fish, might contain high portion of hemoglobin relative to myoglobin. Thus, on average, the heme proteins of sea bass have high tendency to deteriorate compared to that of dark muscle fish like tuna having high portion of myoglobin.

2. Study of some properties of sea bass hemoglobin

The hemoglobin content in sea bass hemolysate was in the range of 83.52 – 90.19 % of total proteins. Thereafter, the hemolysate was defined to hemoglobin. The absorbance spectra (650-350 nm) of sea bass hemoglobin (Figure 8A) possessed the general characteristics of hemoglobin as found in other organisms. It was similar to that of the hemoglobin obtained from fetal and adult human (Watkins *et al.*, 1985; Zijlstra *et al.*, 1991), trout (Falcioni *et al.*, 1978), cod

(Pazos *et al.*, 2005; Thongruang *et al.*, 2006), rainbow trout (Richards and Hultin, 2000), bovine (Richards *et al.*, 2002; Manoz and De Juan, 2007), *Mycobacterium smegmatis* (Lama *et al.*, 2006), and Cyanobacteria (Thorsteinsson *et al.*, 1996). However, there are differences in the wavelength of each peaks, it might be caused from methods of extraction (Gurinovich *et al.*, 1968), environments (Postnikova and Yumakova, 1991), and individual characteristics (Sanna *et al.*, 1997).

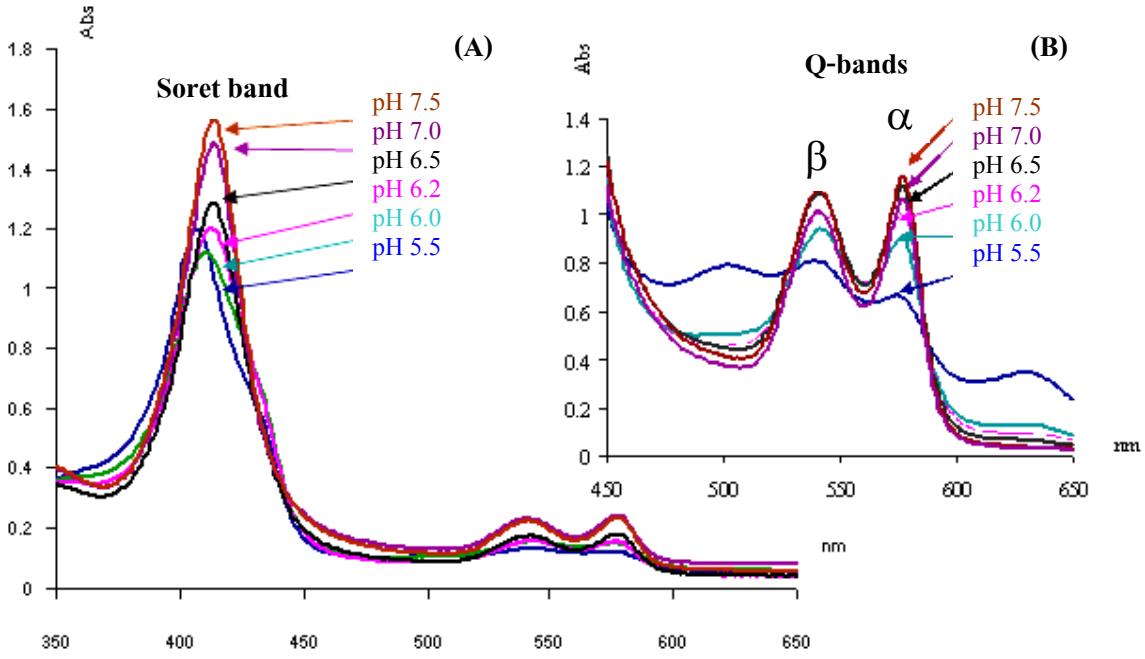


Figure 8 Typical visible light absorbance spectrum of sea bass hemoglobin (5.8 μM) in 50 mM phosphate buffer at pH 5.5 to 7.2. (A) the resolution of Soret peak, 20 min after diluted in phosphate buffer, (B) the Q-bands spectra of sea bass hemoglobin (1.4 μM) after 25 min of dilution, at 15°C.

The spectra of sea bass hemoglobin consisted of three absorbance peaks that is a typical spectrum of metalloporphyrin complexes known as the *etio* spectra. It is due to (a) substitution of H atom at position 1-8 of the porphyrin system by methyl, ethyl, vinyl, propionic acid and acetic acid, (b) four methane bridges, and (c) carboxyl group in conjugation with the porphyrin rings (Marks, 1969). The *etio* spectra were classified into two main bands: Soret band and Q-band. The Soret band shows one absorbance peak in the region of 400 nm while the Q-band are two peaks known as α-band and β-band in the region of 500-600 nm (Marks, 1969).

The absorption spectrum in the Soret region attributed to an essentially pure π-π*porphyrin transition (Jensen *et al.*, 2005). Moreover, the spectra within the Soret region are determined by amino acid residue at which bound by heme group and amino acid sequence in the globular protein (Hsu and Woody, 1971). Whereas, the Q-band transition is contaminated with some charge-transfer character arising from π electron of porphyrin and oxygen to iron transitions that is typical of hexa-coordinate high spin form of hemoglobin (Lama *et al.*, 2006). Binding of oxygen with sixth coordinate ligand of iron modified orientation of the heme group resulting in a red shift of the protein heme spectrum (Zentz *et al.*, 1994; Bauer *et al.*, 1998; Gabbianelli *et al.*, 2004).

The Soret peak of sea bass hemoglobin was observed at 414 nm. The wavelength at peak in the Soret band of sea bass hemoglobin was decreased from 414 nm to 407 nm (~7 nm band shifted) when pH decreased from 7.5 to 5.5 (Figure 8A). The result was to be in line with a blue shift of absorbance spectra of cod hemoglobin due to exposure to pH 5.5 (Thongruang *et al.*, 2006). It needs to be mention that in acidic region the spectrum showed the shoulder at ~450 nm.

The absorbance peaks at 575 and 540 nm (Figure 8B) represent the α-band and β-band, respectively, indicated characteristic of hemoglobin oxygenation. These peaks involved the ligand transition and porphyrin π-π*transitions (Jensen *et al.*, 2005). These maxima are typical coordination of a ligand to the sixth position of the heme-iron represented a low spin heme-iron. Therefore, the photo dissociation occurs via the metal transition through to locate in the red spectral region (Zemer *et al.*, 1966) with a level crossing by exciting into the allowed higher-energy porphyrin of Q-band (Eisert *et al.*, 1979). The difference between absorbance at the peak of 575 nm and that of the valley existed between 575 and 540 nm refracted to a redox state of hemoglobin. The highest difference was observed at pH 7.0 and 7.5, indicated that OxyHb was in the dominant form (Pelster and Decker, 2004). The difference was decreased when the pH was decreased from pH 7.5 to 6.0, suggesting deoxygenation of sea bass hemoglobin (Buaneow *et al.*, 2008).

The ultraviolet-visible spectroscopy (650-350 nm) was used to study the hemoglobin transitions for monitoring of the structural changes (Borgesa *et al.*, 2007). The changes of the Q-bands together with the Soret band are typical for the conversion of the deoxy form of the hemoprotein into oxy form (Andreyuk and Kisel, 1999). It was occurred when oxygen bound to the

heme group then it was a result to the formation of the oxy form, typical forms (Vincent *et al.*, 2002a; Kristinsson and Hultin, 2004; Vitagliano *et al.*, 2004).

The magnitude of the different spectrum in the UV region are believed resulting from changes in quaternary structure (Perutz and Brunori, 1982) such as the positions of aromatic residues of globin (Henry *et al.*, 1985), replacement of distal histidine and arginine by glycine (Korenaga *et al.*, 2000) or threonine (Cutruzzolà *et al.*, 1997), dissociation of dimers (Zhang *et al.*, 1991), the denaturation of hemoglobin and formation of hemichrome (Winterburn, 1990) and subunits (Rachmilewitz, 1969). These are typical leading to the hexa coordination high spin complex of heme-iron with water molecules.

At pH 5.5 (Figure 8B), the spectra was changed and fit into the general spectra of MetHb (Antonini and Brunori, 1971; Richards and Dettmann, 2003; Buaneow *et al.*, 2008). The formation of MetHb at pH 5.5 is accompanied by hypsochromic and blue shift of the spectrum, and broadening of the Soret and Q-bands (Vitagliano *et al.*, 2004). However, it is worth mentioning that the spectra at various pH values were obtained within 30 min after pH adjustment. Thus, it is possible that increasing incubation time may involve on alteration of absorbance spectra of the hemoglobin at other pH. The absorption spectra of porphyrin IX complex in blue region are pointed to aggregation or denaturation of the porphyrin IX molecules (Herskovits and Solli, 1975). Also the increase in the number of monomer or dimer of hemoglobins present as a cause of the Q-bands broadening. Therefore the lacking of prosthetic group was occurred when is not linked to the protein and the heme groups in this spectra range (Manoz and De Juan, 2007).

Figure 9 illustrates the relative oxygenation of the sea bass hemoglobin with decreasing pH from 7.5 to 5.5. The pH range was chosen since it encompasses the pH values found in post mortem fish. The percent relative oxygenation was decreased with a sigmoidal fashion. This unique is commonly found in other fish hemoglobin, such as skipjack tuna (Jensen, 2001), trout (Richards *et al.*, 2002), cod and herring (Thongruang *et al.*, 2006). But the oxygenation profile was different from the hemoglobin of cow and chicken (Richards *et al.*, 2002). The sigmoidal curve of hemoglobin suggested that the binding of oxygen molecules to the four hemes is all-or-none oxygen cooperation. The first oxygen attaches to the heme very weakly, whereas the others are easier and more strongly binding to the heme (Jensen, 2004).

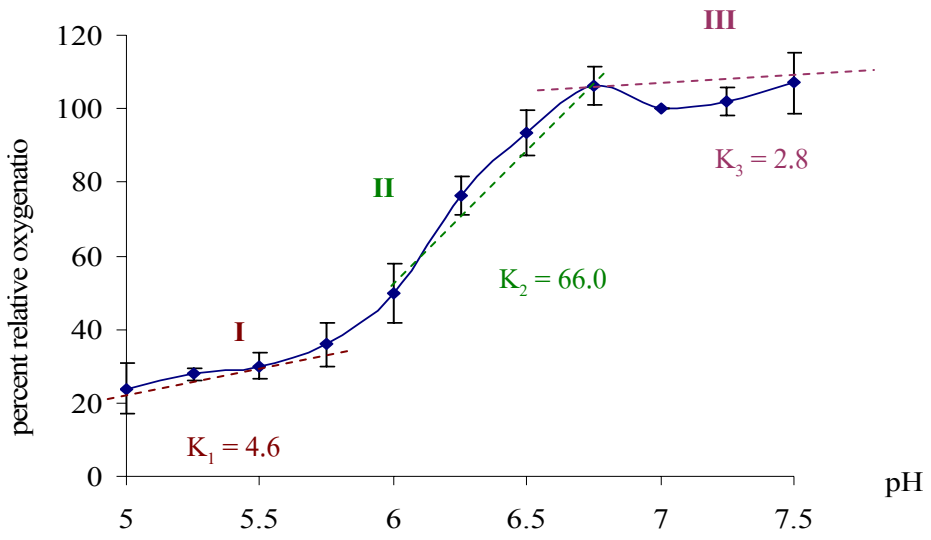


Figure 9 Relative oxygenation of sea bass hemoglobin at various pH values, after 20 min of dilution in phosphate buffer and the final concentration was 5.8 μM

The S-shaped curve could be classified into three significant intervals. There were interval I, II and III with different K_{co}, (Δrelative oxygenation/ΔpH) values, with 4.6, 66.0 and 2.8, respectively. The K_{co} represented the ability of hemoglobin in cooperation of oxygen. The first interval (pH 5.0-5.75) was in the acidic conditions. Interestingly, the relative oxygenation was not lower than 20 %, this might due to containing of having oxygenation independence on pH changes (Weber, 1990). The relative oxygenation of sea bass hemoglobin sharply decreased when pH decreased from 6.5 to 6.0 (interval II). This may be associated with an adaptation of the fish to release the enough oxygen for the normal and emergence situations (Riggs, 1970). The 100% relative oxygenation of sea bass hemoglobin was in the third interval (pH 6.75 – 7.5). The pH-dependent oxygenation is a property of anodic hemoglobin containing titratable components. Moreover, Lowe (1999) reported that the relative oxygenation of hemoglobin was minimized in the strong acid or alkaline pH solutions, because of the denaturation of hemoproteins and porphyrins. (Lowe, 1999)

In the physiological conditions, the (de)oxygenation of hemoglobin is controlled by the bicarbonic-carbonate buffering system. The antagonist of hydrogen ions and oxygen molecules equilibria could be explained by the Bohr effect (Riggs, 1970). When the pH changed,

the H^+ concentration of solvent may be affected to the destruction and formation of salt bridge among hemoglobin subunits (Jensen, 2001). Thus, the proton binding properties of the charge group of the amino acids determined the contributed residues (Tanford, 1982).

Histidine is likely a site for the binding of Bohr protons (Berenbrink *et al.*, 2005). Only the proteins having the usual distal (E7) histidine can manifest a strong proton-catalysis in the autoxidation reaction (Shikama and Matsuoka, 1986; Shikan and Sobajim, 1997; Tada *et al.*, 1998; Suzuki *et al.*, 2000). In general, the hemoglobin with highly amount of histidine residues has the high buffering capacity, thus the proteins tolerance to the environment pH changes. This characteristic was also found in the terrestrial hemoglobins, including that of adult human (Shikama, 2006), chicken, and cow (Richards and Hultin, 2002). Most fish hemoglobins contain lower number of histidine residues than terrestrials. When the pH changed over the tolerance of the protein buffering activity, the breaking down or the forming of some salt bridge and H-bond was likely occurred and resulted in the molecular conformation changes (Jensen, 2004). In the oxygenated state of fish hemoglobin, the carboxyl group of histidine at HC position 3 in the β-chain formed a salt bridge with the side chain of α at lysine in C helix at position 5 (Perutz and Brunori, 1982).

The result suggested that pH, an allosteric effecter on hemoglobin oxygenation, plays a role on determination of oxygen affinity of hemoglobin. Therefore, the concentration of H^+ related to the pH values should affect to the hemoglobin (de)oxygenation in differences rate of oxygen occupy.

3. Effect of pH and ATP on sea bass hemoglobin

The time-oxygenation curve of sea bass hemoglobin at various pH values is shown in Figure 10. Obviously, the deoxygenation rate increased with decreasing of pH, especially within the first 120 min. The relative oxygenation of sea bass hemoglobin at pH 7.0 was in a constant rate within 360 min. It was proposed that modification of hemoglobin conformation was stimulated by an increase concentration of hemoglobin (Tsuruga and Shikama, 1997). The percent relative oxygenation of sea bass hemoglobin was level off after 120 min of exposure to lower than 7.0. This might be originated from the limiting of proton concentration.

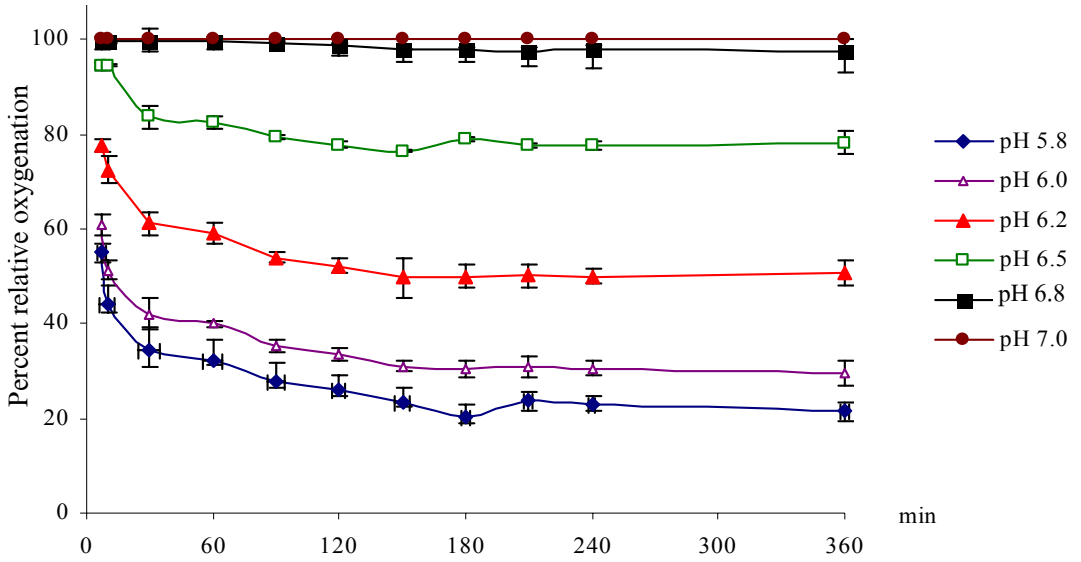


Figure 10 Effect of pH on the percent relative oxygenation of sea bass hemoglobin (5.8 μM) at 15°C, within 360 min

After addition of ATP for 30 min, deoxygenation of hemoglobin was noticed in all samples, excepted at pH 6.2 (Table 4). The decreasing in the percent relative oxygenation of hemoglobin at pH 7.0 with added ATP confirmed that ATP was able to stimulate deoxygenation of hemoglobin. There was no significant difference between the percent relative oxygenation of hemoglobin at pH 6.2 with and without added ATP suggested that ATP was less effect allosteric modulator relative to that of pH. However, at pH 6.5 ATP could enhance the effect of pH on decreasing oxygenation of hemoglobin. Thus, the result confirmed that of fluid blood riched of OxyHb and ATP and its derivertives during postmortem process event just would increase concentration of DeoxyHb.

Table 4 Effect of pH and ATP on relative oxygenation of sea bass hemoglobin (mean ± SD)

ATP (mM)	Percent relative oxygenation		
	pH 6.20	pH 6.50	pH 7.00
0.00	59.09±2.38 ^{aw}	92.47±1.52 ^{bw}	100.00±0.00 ^{cw}
0.50	54.90±2.58 ^{aw}	88.76±2.21 ^{bx}	93.57±2.59 ^{cx}
1.00	52.92±1.97 ^{aw}	88.14±1.02 ^{by}	91.09±3.28 ^{bcy}
2.00	52.32±2.14 ^{aw}	86.94±2.09 ^{bz}	89.65±5.50 ^{cz}

* The means followed by different letters in the same row (a-c) or column (w-z) are significantly difference ($p<0.05$).

Shikama (2006) reviewed that when the pH was lowered to the acidic condition, the hemoglobin was changed from relaxed to tensed state or OxyHb to DeoxyHb. In the relaxed form, an oxygen atom formulated a bonding to the proximal heme-iron and a hydrogen bond with the distal histidine (E7) at the ε-N position (Shikama, 2006). In contrast to a decrease of pH, ATP affected to the hemoglobin oxygenation by the formation of the salt bridges to some amino acids at the interface in the central cavity between two β chains (Perutz and Brunori, 1982). The positive charge residues involved in phosphate binding at the central cavity may acts as a reverse Bohr groups in fish hemoglobins (Fago *et al.*, 1995).

4. Effect of pH, washing, and rigor-mortis stages on pro-oxidant properties of hemoglobin

4.1 Effect of pH and washing on lipid oxidation

The storage time before TBARS value reaching over 15-20 μmole MDA (7-8 mgMDA/kg) or "lag time" was used to monitor the oxidative stability of fish muscle (Richards and Li, 2004). In this primliminary experiment, the content of thiobarbituric reactive substances (TBARS) about 15-20 μmole MDA/kg was found to be a critical value for sensory acceptance limiting. The TBARS development of unwashed sea bass mince with and without added sea bass hemoglobins at pH 6.2, 7.0 and 6.5 (physiological pH) is shown in Figure 11. It was found that the lag time of development of lipid oxidation in trout at pH 6.0 with added hemoglobin was 12 h (Richards and Hultin, 2000).

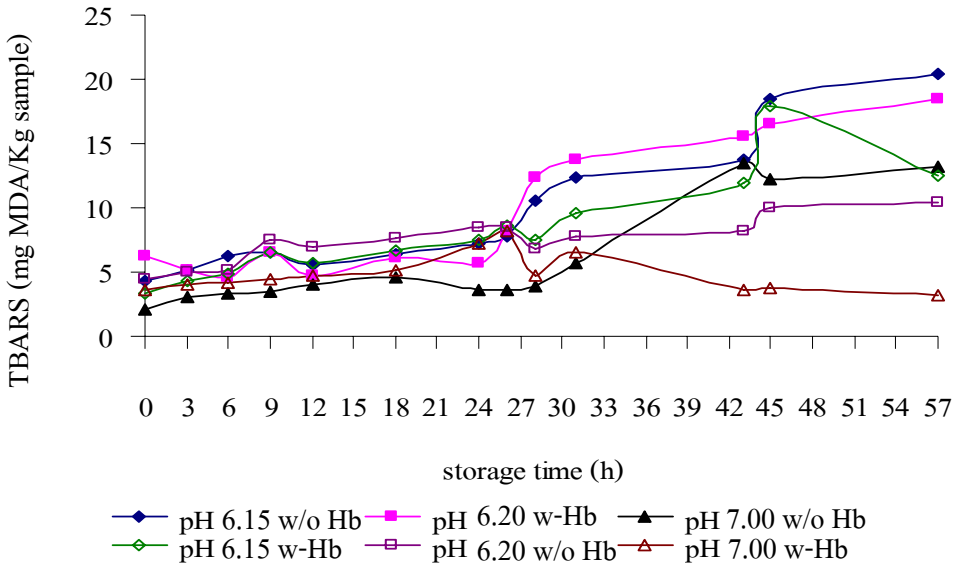


Figure 11 Development of TBARS value of unwashed sea bass mince with (w-Hb) and without (w/o Hb) the addition of sea bass hemoglobin (5.8 µmole/kg muscle) at pH 6.20, 7.00 and unadjusted pH.

Lipid oxidation, according to the TBARS values, occurred readily in the unwashed mince regardless of an addition of hemoglobin (Figure 11). The addition of hemoglobin showed decrease the lag time of oxidative stability in the unwashed sea bass mince. The lag time of washed minces at pH 6.2 and 6.0 (unadjusted pH) mince was decreased from 26 and 28 h to 24 and 26 h, respectively, after hemoglobin was added

DeoxyHb and MetHb were proposed to be potential pro-oxidants in muscle based foods (Gutteridge, 1987). Richards *et al.* (2002) reported that the trout hemoglobin stimulated lipid oxidation at pH 6.0 corresponding to increasing of its deoxy-form. There are many possible situations leading to exposure of hemoglobin to deoxygenation by inducing factors such as low pH and high ATP during a rough handling of fish in board vessels and postmortem process. This study proved that deoxygenation of sea bass hemoglobin was stimulated by mainly lowering of pH and exposure to ATP although with less efficacy in later one.

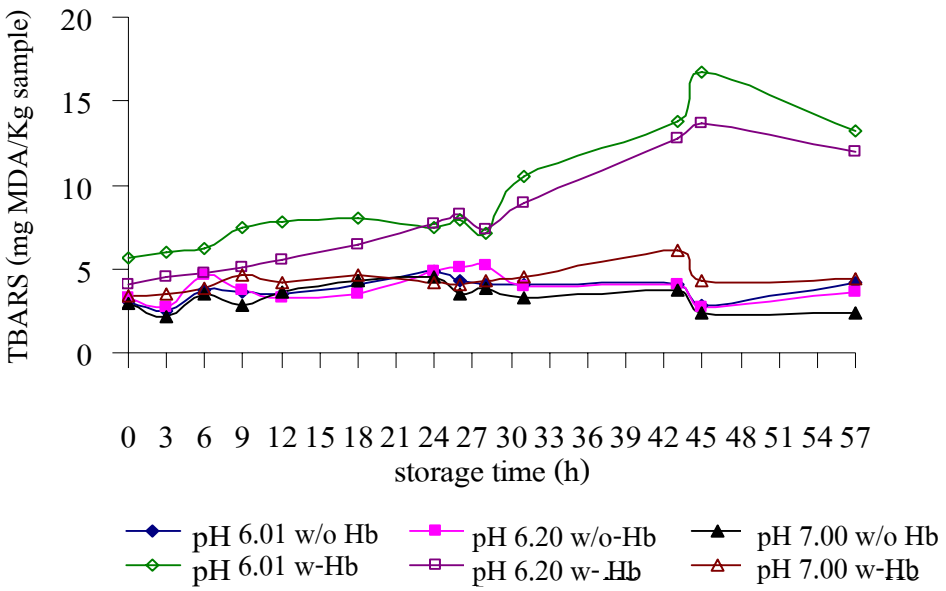


Figure 12. Development of TBARS value of washed sea bass mince with (w-Hb) and without (w/o Hb) the addition of sea bass hemoglobin (5.8 µmole/kg muscle) at pH 6.20, 7.00 and physiological pH.

Washing could extend the lag time of lipid oxidation in all treatments reflected by an extention of lag time over 60 h. The lag times of unwashed minced and washed mince with added hemoglobin at pH 6.2 were equivalent. This implied that the hemoglobin played a primary role on lipid oxidation in sea bass muscle. Additionally, decline in pH during the post mortem development may enhance deoxygenation of hemoglobin (Bonafe *et al.*, 1999). Based on the finding of section 2.2, the sea bass had low relative oxygenation at pH 6.2. Thus, the results revealed that pro-oxidative activities of the hemoglobin were associated with its deoxygenation with similarity to finding of Binotti *et al.* (1971) and Shikama and Matsuoka (1986). It was reported that the rate of lipid oxidation in washed muscle system was affected by the hemoglobin (de)oxygenation propertie. For example, the Bohr effect of cod hemoglobin was stronger than that of trout, chicken, and bovine hemoglobin resulting in a hgher rate of lipid oxidation with existence of cod hemoglobin relative to that with an existence of those hemoglobin (Richards *et al.*, 2002). DeoxyHb caused more 3.5 ties peroxidative activity than OxyHb and MetHb (Pietrzak and Miller, 1989). In addition, DeoxyHb autoxidizes faster than OxyHb that involved with the spin state of the iron atom inside the heme ring (Livingston and Brown, 1981). The ferrous iron atom of DeoxyHb

(Fe^{2+}) is a 5-coordinated complex where the iron has 4 bonds to the porphyrin heme ring and 1 bond to a histidine residue of the globin. This causes the iron to be in a high spin state and hence highly susceptible to oxidation to ferric MetHb (Fe^{3+}). The iron atom of OxyHb (Fe^{2+}) is a 6-coordinated complex with an additional ligand to O_2, which causes the iron to be in a low spin state and less susceptible to oxidation. From the literatures, lowering of pH is known to be enhancing of (i) hemin release (Hargrove *et al.*, 1996a), (ii) solubility of iron released from hemoglobin (Schafer and Buettner, 2001), (iii) acid-catalyzed hemoglobin autoxidation (Shikama, 1998) and (iv) formation of H_2O_2 from O_2^- (Halliwell and Gutteridge, 1989), which are all parameters known to stimulate lipid oxidation (Morissey *et al.*, 1998).

4.2 Post-rigor mortis stages of sea bass as measure by firmness

The firmness values (g) of dorsal part of sea bass after death are shown in Figure 13. The changing in firmness of sea bass could be devided into 3 stages. Within 4 hours after death (pre-rigor mortis stage), the firmness gradually increased from 50 to 90 g. After death for 5 h, the firmness increased from 100 g and reached the maximum value of 115 g at 8 h which classified as rigor-mortis period. This result agreed with Siripongvuttikorn (2004) who reported that the post-rigor mortis stage of sea bass began at 8 hour after death. Greaser and Pearson (1999) reported that course of completion of rigor mortis in the fish required 5-24 h. The evolution of postmortem pH depends on the carbohydrate reserves (glycogen) of the fish (Lowe, 1992) as well as the degree of stress suffered prior to slaughter (Azam *et al.*, 1989; Lowe *et al.*, 1993). Olafsdottir *et al.* (1997) mentioned that the firmness was correlated to the degradation of ATP and its derivatives during the rigor process (Olafsdottir *et al.,* 1997).

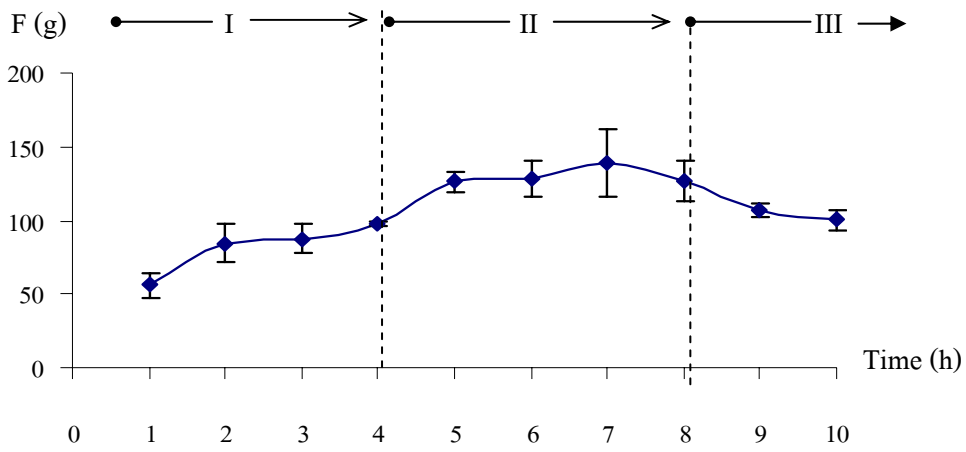


Figure 13 Firmness of dorsal part muscle during the rigor development of sea bass fillet. Where, the state I, II and III represented to the stage of pre-rigor, rigor and post-rigor mortis, respectively.

4.3 Effect of rigor stage, pH, washing and hemoglobin on lipid oxidation in sea bass mince

The sea bass at pre-rigor, rigor, and post-rigor stage as evaluated by firmness values were used for the experiments. It was unexpected results that pH values of these different post mortem stages of the fish were quite low and there was no significant different (Table 5). Althogh, the marginal difference among the pH values of the fish at stage II and III was significant. It is difficult to give a clear explaination for the observation. However, the similar pH of the pre-rigor sea bass was also reported by Siripongvutikorn (2005). Since, the fish used for the experiment was occasionally disturbed while fisherman caught the fish for selling. This might create stress and accumulation of acid in the remaining fish. Moreover, white flesh fish is known for its low buffering capacity relative to that of dark flesh fish (Sohn *et al.*, 2005).

Lipid hydroperoxide values (LHP) of the minced fish obtained from each post mortem stages were not significant difference. Whereas, TBARS value was significant elevate with the increasing of post mortem aging. It was proposed that the TBARS was more correlated to the storage age or deterioration and more reliable than LHP. This may partially due to LHP is primary products of lipid oxidation. Thus, LHP could be transformed into other products causing fluctuation in its content (Richards and Dettmann, 2003). However, a benefit of the LHP was important to

identify the initial quality of fish flesh (Chan *et al.*, 1997; Richards and Dettmann, 2003; Richards and Li, 2004).

Table 5 pH and lipid oxidation status of post mortem sea bass muscle (dorsal part)

Stage	**I**	**II**	**III**
Time of sampling (h after death)	2	10	48
pH	6.15 ± 0.01^{a}	6.17 ± 0.09^{ab}	6.30 ± 0.04^{b}
Lipid hydroperoxide value[1]	121.39 ± 22.07^{a}	113.04 ± 12.00^{a}	112.55 ± 45.27^{a}
TBARS value[2]	4.30 ± 0.30^{a}	5.23 ± 0.20^{b}	6.89 ± 0.40^{c}

[1] Lipid hydroperoxide value expressed as μmole cumene hydroperoxide/kg sample

[2] TBARS value reported in the unit of mg MDA/kg sample

Table 6 and 7 showed development of lipid oxidation in sea bass mince with various conditions. LHP of 200 μmole cumene LHP/kg muscles and TBARS value of 15 mg MDA/kg muscles were used to monitoring the stability of lipid oxidation (Richards *et al.*, 2002).

The preformed LHP content was not significant difference among the minces of post mortem stages. Whereas, TBARS values of mince of stage I and II were significant lower than that of mince from stage III. This might cause of the result from chain reaction and dismutation of LHP, thus decreasing in preformed LHP corresponsding to the increasing in TBARS. It is important to note that the preformed LHP in unwashed minces without added hemoglobin obtained from different post mortem stages were similar, excepted the stage I. In another words, the marginal amount of LHP was produced by extension of post mortem storage from 2 h of stage I to 48 h of stage III. This was likely due to the intact structure of muscle tissue that prevented endogenous pro- and anti-oxidation involving in the lipid oxidation. However, when the tissue was minced the significant difference in lag times was noticed. This would be due to release of either pro- or anti-oxidative components from damaged cell. Thus, decreasing in lag time of unwashed mince without added hemoglobin obtained from the stage III suggested high proportion of pro-oxidative component in the fish. There is an increase in pro-oxidative substances such as hemin and low molecular weight iron (Decker and Hultin, 1990), lipid peroxides (Hardy and Smith, 19676), and H_2O_2 (Harel and Kanner, 1985) during the post mortem process.

Table 6 Effect of pH, hemoglobin addition and rigor mortis stages on lag time (h) of LHP values elevated to 200 μmole cumene lipid hydroperoxides /kg of washed and unwashed sea bass minced

Rigor stage	Unwashed mince			Washed mince		
	pH	Without Hb (LHP*)	With Hb (LHP*)	pH	Without Hb (LHP*)	With Hb (LHP*)
Stage I	6.15*	24(121.39±22.07)	26 (118.64±14.25)	6.01*	>60 (66.17±11.07)	24(92.24±12.05)
	6.20	24(123.00±12.82)	24(127.05±35.66)	6.20	>60(78.41±24.61)	24(73.62±18.40)
	7.00	31(91.20±30.46)	28(60.13±15.02)	7.00	>60(66.97±22.02)	>60(75.88±24.21)
Stage II	6.17*	26(113.04±12.00)	6(106.40±01.24)	6.09*	>60(130.60±12.05)	6(102.60±26.53)
	6.20	9(106.42±29.01)	6(108.47±12.51)	6.20	>60(111.24±14.00)	8(107.89±12.70)
	7.00	24(115.66±17.23)	30(98.83±40.87)	7.00	>60(96.74±29.35)	>60(93.53±32.52)
Stage III	6.30*	9(112.55±45.22)	7(134.86±16.57)	5.98*	>60(135.60±14.23)	7(116.34±11.06)
	6.20	9(143.95±32.54)	7(110.38±22.95)	6.20	>60(120.35±10.12)	7(110.61±10.57)
	7.00	9(115.67±11.08)	9(74.13±14.28)	7.00	>60(61.04±14.76)	>60(98.59±22.02)

* the unadjusted pH

LHP* represents the lipid hydroperoxide (μ mole cumene LHP/kg sample) at the initial time

Washing showed beneficial effect on removal of preformed LHP only in the mince obtained from the fish at stage I. In the case of TBARS, washing could remove the preformed TBARS component in fish muscle of all post mortem stage. The extractable TBARS was, however, marginal. The results thus implied that significant portion of the preformed primary and secondary oxidation products might associate with insoluble fraction of fish tissue. This explanation would support the shortening in TBARS lag time of the fish minces obtained from aged fish mince containing high amount of oxidation products. The result suggested that washing should be performed in pre rigor fresh fish in order to effectively remove endogenous oxidation product. Richards and Li (2004) reported that washing improved quality of fish fillet obtained from the stage I where its blood remained fluid.

Table 7 Effect of pH, hemoglobin addition and rigor mortis stages on lag time (h) of TBARS values elevated to20 μmole Tetramethoxypropane/kg of washed and unwashed sea bass minced

Rigor stage	Unwashed mince			Washed mince		
	pH	Without Hb (TBARS*)	With Hb (TBARS*)	pH	Without Hb (TBARS*)	With Hb (TBARS*)
Stage I	6.15*	28(4.43±0.34)	26(3.37±0.74)	6.01*	>60(3.01±0.07)	18(5.63±0.11)
	6.20	26(4.28±0.15)	24(6.17±0.23)	6.20	>60(3.26±0.40)	26(4.10±0.56)
	7.00	43(2.11±0.72)	26(3.58±0.86)	7.00	>60(3.00±0.37)	>60(3.41±0.08)
Stage II	6.17*	26(5.23±0.20)	8(5.90±0.18)	6.09*	>60(3.55±0.04)	8(5.70±0.24)
	6.20	9(5.65±0.41)	8(5.88±0.02)	6.20	>60(3.62±0.38)	8(4.72±0.32)
	7.00	24 (5.05±0.25)	30(4.93±0.74)	7.00	>60(3.45±0.01)	>60(2.68±0.11)
Stage III	6.30*	12(6.89±0.40)	6(3.63±0.47)	5.98*	30(5.93±0.01)	9(4.46±0.43)
	6.20	9(3.08±0.51)	7(4.21±0.31)	6.20	54(3.15±0.12)	9(5.65±0.27)
	7.00	12(4.73±0.69)	12(5.28±0.08)	7.00	>60(3.09±0.19)	>60(4.81±0.18)

* the unadjusted pH

TBARS* represents the TBARS (mg MDA/kg sample) at the initial time

Sea bass hemoglobin showed the antioxidative activity in unwashed sea bass at stage I fish. This might cause the synproportionation of hemoglobin (Giulivi and Davies, 1990). Whereas, the hemoglobin acts as pro-oxidant in the stage II and III unwashed mince. However, sea bass hemoglobin possessed pro-oxidant function in washed sea bass mince of all post mortem stages. Based on the lag time of LHP and TBARS development, addition of hemoglobin into either washed or unwashed mince at pH 6.15 and 6.20 stimulated effectively lipid oxidation especially in washed mince. This observation implied that the pro-oxidative status of the mince system would increase with the existence of hemoglobin at low pH. It was important to note that a large reduction in lag time of LHP and TBARS were found in unwashed systems obtained from the fish at stage II. It was likely that at stage I significant amount of endogenous anti-oxidative components would remain and capable to retard pro-oxidative activity of hemoglobin. In contrast with fish at stage III, where the endogenous pro-oxidation like an addition of hemoglobin would show limited effect on stimulation of lipid oxidation.

5. Evaluation of antioxidative properties of galangal and lemon grass extracts

The polypolyphenolic content and some antioxidative properties of galangal and lemon grass extracts obtained by various ethanol concentrations are shown in Table 8. Total polypolyphenolic content of the extracts was quantified using gallic acid and catechin as standards. Although the total polyphenolic contents of the extracts determined by using different standard reagents were different. There showed the similar trend respected to the effect of ethanol concentration. Thereafter, the total polyphenolic compound content was calculated base on the gallic acid equivalent (GAE).

Galangal extracts had polyphenolic content significantly higher than that of lemon grass extracts, excepted the E00 and E95 treatments, (Table 8). This observation was in accordance with the results of Jantachote *et al*. (2006) who reported that the ethanolic extraction yield of galangal (range: 1000-3000 mg/100 g) was higher than that of lemon grass (range: 780-885 mg/100 g). Additionally, it was found that total extractable polypolyphenolic content of both plants increased according to increasing of ethanol concentration up to 50 % (v/v). However, an increase of the ethanol concentration to 95% (v/v) decreased extraction yields. The results suggested that extractability of polyphenolics determined by polarity of the solvent. This may associate with non polar and polar proportion and distribution within each polyphenolic compounds (Savova *et al.*, 2007) resulting in their affinity to the solvent (Kang *et al.*, 2002). The mixture of ethanol and water makes the large polarity range of solvent. The highest extractability of polyphenolic would be obtained by using the solvent having polarity matched to that of the phenolic substances. Rukachaisirikul and Dampawon (1994) found that mainly antioxidant compounds in the fresh extract of galangal were chavicol, chavicol acetate, 1-hydroxy -chaviocol acetate, acetoxycinnamyl alcohol, and acetoxycinnamyl acetate. Whereas, Cheel *et al.* (2005) found that isoorientin, isoscoparin, swertiajaponin, isoorientin 2' '-*O*-rhamnoside, orientin, chlorogenic acid, and caffeic acid, were isolated and identified as the major compounds in lemon grass. This result agreed with Savova *et al.* (2007) who found that 50% v/v ethanol of grape seed obtained as the polarity comparable with the polarity of polyphenolic compounds in the sample.

Table 8 Polypolyphenolic content and antioxidant properties of galangal and lemon grass extracts with various concentration of ethanol solution

Treatment	Polyphenolic content (mg /100 g Wb^{-1})		DPPH radical scavenging activity IC_{50}		Maximum percent Fe^{2+} chelating (%)
	gallic acid	catechin	mg Db	mg GAE	
GE 00	4.86 ± 0.07^{bc}	3.91 ± 0.04^{bc}	4.31 ± 0.37^{e}	0.95 ± 0.08^{cde}	73.77 ± 2.11^{ef}
GE 35	5.37 ± 0.37^{cd}	4.33 ± 0.28^{cd}	3.93 ± 0.07^{e}	0.95 ± 0.02^{d}	71.61 ± 0.29^{e}
GE 50	8.87 ± 0.64^{f}	7.16 ± 0.50^{f}	3.03 ± 0.06^{bc}	1.22 ± 0.02^{e}	72.95 ± 3.49^{ef}
GE 75	8.79 ± 0.37^{f}	7.09 ± 0.28^{f}	2.46 ± 0.23^{a}	0.98 ± 0.09^{cd}	69.51 ± 3.73^{e}
GE 95	3.07 ± 0.11^{abc}	2.46 ± 0.07^{abc}	2.57 ± 0.06^{a}	0.36 ± 0.01^{a}	78.12 ± 1.75^{f}
LE 00	4.85 ± 0.08^{bc}	3.91 ± 0.05^{bc}	9.89 ± 0.58^{f}	1.71 ± 0.10^{f}	27.25 ± 2.98^{a}
LE 35	4.47 ± 0.47^{b}	3.60 ± 0.36^{b}	4.06 ± 0.37^{c}	0.65 ± 0.06^{bc}	40.72 ± 3.70^{b}
LE 50	5.63 ± 0.08^{e}	4.54 ± 0.05^{e}	3.41 ± 0.08^{ab}	0.69 ± 0.02^{c}	55.94 ± 3.44^{c}
LE 75	5.33 ± 0.08^{cd}	4.29 ± 0.05^{cd}	3.36 ± 0.18^{ab}	0.64 ± 0.04^{c}	62.75 ± 2.24^{d}
LE 95	3.47 ± 0.16^{a}	2.79 ± 0.11^{a}	3.87 ± 0.08^{bc}	0.48 ± 0.01^{b}	70.99 ± 1.02^{e}

Mean ± SD from triplicate determinations

The different superscripts in the same column denoted the significances ($p<0.05$)

Abbreviation: GE00, GE35, GE50, GE75 and GE95 represented the galangal extracts obtained by using 0, 35, 50, 75 or 95% of ethanol, respectively. LE00, LE35, LE50, LE75 and LE95 represented the lemon grass extracts obtained by using 0, 35, 50, 75 or 95% of ethanol, respectively.

Temperature was another factor affecting extraction yield (Richter *et al.*, 1996). Generally, increasing of temperature increased the solubility and diffusibility of the phenolic compound (Cacace and Mazza, 2006). However, Jantachote *et al.* (2006) reported that there was no significant of temperature between 65-75 ˚C on antioxidant activity of galangal and lemon grass extracts.

It is worth mentioning that the extracts of both spices obtained with 95% ethanol changed to be the rubbery material after evaporation. This problem was solved by adding small amount of ethanol before the addition of water. However, this treatment caused some sedimentation

of solids like starch. These problems were also noticed in parsley leaves and cardamom (Hinneburg *et al.*, 2006) and in galangal (Rukachaisirikul and Dampawon, 1994).

The DPPH radical scavenging activity of the galangal and lemon grass extracts is shown in the Table 8. Ascorbic acid, BHA, BHT and gallic acid were used as references and showed IC_{50} values for DPPH radical scavenging activity of 0.12 ± 0.01, 0.20 ± 0.01, 0.24 ± 0.01 and 0.08 ± 0.01 mg, respectively. Based on their dry weight basis (Db) the DPPH radical scavenging activity of the galangal extracts was obviously higher than that of lemon grass extracts. The GE95 and LE95 showed greater activity than that of other extracts whereas the L00 was the lowest activities. However, the DPPH radical scavenging activity of lemon grass and galangal extracts did not linear correlated to polyphenolic content. This may due to different polarity of compounds in the extract that provided different preferential activity. The DPPH radical scavenging activity of the extracts increased when concentration of ethanol solution was higher than 35% v/v. Polyphenolics were able to be antioxidant due to the presence of hydroxyl substituents and their aromatic structure, which enable them to scavenge free radicals (Verhagen *et al.*, 1997; Rigo *et al.*, 2000; Kefalas *et al.*, 2003). Cheel *et al.* (2005) suggested that 76% of the free radical scavenging activity of the lemon grass extracts resulted from the contribution of flavonoids. However, the DPPH radical scavenging activity was better in the ethanolic or methanolic extracts than in aqueous extract. This might due to an appropriate media for dissolving DPPH radical with respect to water (Saito and Kawabata, 2004). Dangles *et al.* (2000) also reported that medium was an important factor influencing the reaction of polyphenols with DPPH radical.

Table 8 also shows the iron chelating activity of galangal and lemon grass extracts as compared to the chelating activity of ethylene diamene tetraacetic acid (EDTA). The highest chelating activity of galangal extracts was obtained by using 95% (v/v) ethanol. Similar to the chelating activity of lemon grass extracts increased with the increasing of ethanol concentration. However, lemon grass extract showed lower chelating acticity compared with galangal extract at the similarity ethanol concentration. In comparison, the LE00 was the lowest chelating activity whereas the GE95 was the best activity among all treatments. This was contrast to the lemon grass extract where its chelation activity of lemon grass extracts was more correlated (R^2= 0.9706, $p<0.05$) to the ethanol concentration than that of galangal extracts (R^2= 0.0709, $p<0.05$). The result suggested that water might be economical solvent for extraction of galangal to obtain natural metal

chelating agent. Certain phenolic compounds have properly oriented functional groups, which can chelate metal ions (Thompson and Williams, 1976). For example, van Acker *et al*., (1996) reported that chelating capacity of iron (II) by flavonoids ranged from strong to weak depending on their molecular structural feature. Thompson and Williams (1976) found that the stability of the metal-antioxidant complex was higher in six-membered than five-membered ring complexes. The ratio of hydrophilic to hydrophobic phenols was determined the chelation activity in each plant differently (Wettasinghe and Shahidi, 2002).

6. Effectiveness of galangal and lemon grass extracts on retardation of lipid oxidation mediated by sea bass hemoglobin

The effectiveness of galangal and lemon grass extracts on retardation of lipid oxidation in sea bass minced was schemed in Table 9. Based on thelag time, the Ctrl+Hb (24) and LE00 (6) revealed faster TBARS development rate than that of other treatments. In contrast, the lag time of GE95 and LE95 treatment were over 180 h. The effectiveness in retardation of TBARS development increased with increasing of ethanol concentration. However, the GE75 and LE75 were comparable in the oxidative retardation activity. The galangal extracts showed the better activity than did the lemon grass extracts. Nevertheless, the LE00 showed a pro-oxidant activity with the lag time of 6 h resulting in a higher rate of the lipid oxidation than that of Ctrl+Hb. This might be due to the presence of chlorophyll, and pheotophytin in this extract (Usuki *et al.*, 1984).

Lag time of galangal extract in all treatments was unlikely correlated to the chelating activity (R^2 = 0.5105, $p<0.05$)(see Table 8). Whereas, the lag time of lemon grass extract was obviously correlated to the chelating activity (R^2= 0.9938, $p<0.05$). This might due to different of active compounds corresonponding to their funtionallity. Cheah and Abu-Hasim (2000) reported that galangal extract at 5 and 10 % (w/w) delayed development of lipid oxidation in raw beef comparable with that of 0.02% BHT. Moreover, it was reported that polyphenolic compounds, such as epigallocatechin gallate (a member of hydroxyl -pyranone) were efficient in deactivating pro-oxidant activity of ferryl myoglobin (Hu and Skibsted, 2002). It was proposed that phenolic compounds possessed a structure like gallic acid containing three –OH groups in the B ring demonstrated high antioxidative activity (Villaño *et al.*, 2005).

Table 9 Effect of galangal and lemon grass extracts on lag times of TBARS development in washed sea bass mince

Treatment	Lag time (h)	TBARS*	Treatment	Lag time (h)	TBARS*
Ctrl-Hb	126	7.44±0.36	Ctrl+Hb	24	10.45±5.64
GE00	48	6.96±3.08	LE00	6	9.34±0.25
GE35	156	6.15±1.01	LE35	96	7.56±0.90
GE50	168	7.59±0.46	LE50	120	6.02±1.59
GE75	180	7.08±3.47	LE75	180	7.35±0.88
GE95	> 180	6.86±0.43	LE95	>180	6.36±0.84

TBARS* at the lag time, mg MDA/kg sample

Abbreviation: GE00, GE35, GE50, GE75 and GE95 represented the galangal extracts obtained by using 0, 35, 50, 75 or 95% of ethanol, respectively. LE00, LE35, LE50, LE75 and LE95 represented the lemon grass extracts obtained by using 0, 35, 50, 75 or 95% of ethanol, respectively.

Mean ± SD from triplicate determinations

The different superscripts in the same column denoted the significances ($p<0.05$)

There are relatively a few reports that studied the effect of polyphenolic compounds on a pro-oxidant activity of hemoglobin. Xi and Guo (2007) studied on the association of flavonoids with hemoglobin and the effects of liposomes on the binding of flavonoids to hemoglobin. It was found that upon binding with flavonoids the conformation of hemoglobin was changed with increasing of the polarity around the tryptophan residues and decreasing of the hydrophobicity. The other studies reported interaction of a conformational change of hemoglobin with the loss of helical stability due to binding with flavonoids (Zhang *et al.*, 2004; Roth *et al.*, 2005; Xi and Guo, 2007). The deoxygenation curve of sea bass hemoglobin is shown in the Figure 14. The result showed effectiveness of galangal (GE95) extract on retardation of DeoxyHb formation during 10 min of observation. The lowering of the relative oxygenation of sea bass hemoglobin added with GE95 was comparable with that of gallic acid. The observation may be due to two possible reasons; (a) addition of alcohol may decrease the hemoglobin solubility and/or (b) the interaction between phenolics and hemoglobin molecules may develop (Outtrup *et al.*, 1987).

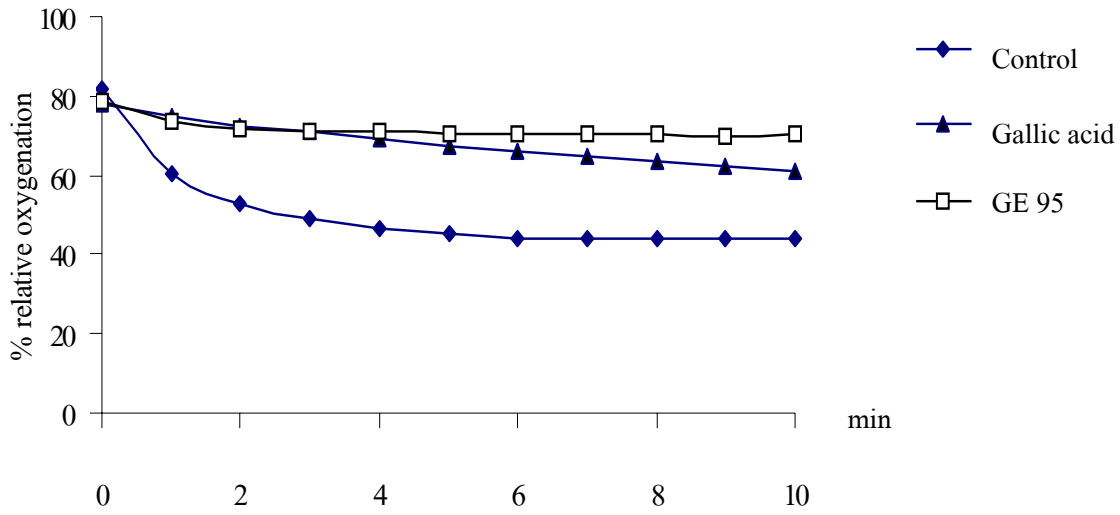


Figure 14 Comparison of the sea bass hemoglobin (5.8 μM) deoxygenation at pH 6.5 with the addition of 30 % ethanol (control), gallic acid (5.8 μM) and GE95 (equivalents to 5.8 μM gallic acid)

It was well known that pH could determine the interactions of phenolics and proteins (Hurell and Finot, 1984). There are 5 potential types of interactions of phenolic and proteins: hydrogen bonding, π-bonding, and hydrophobic, ionic, and covalent linkages (Hagerman, 1992; Bianco *et al.*, 1997). The main mechanisms of protein polyphenol interaction at moderate pH are thought to be governed by π-bonding (Bianco *et al.*, 1997). Kroll and Rawel (2001) reported that the gallic acid, o-, and p-hydroxyphenols, and p-quinone showed high activity leading to structural changes in myoglobin, especially tryptophan residue. Goupy *et al*. (2007) proposed the mechanism and synergism of α-tocopherol and quercetin as inhibitors of the heme-induced peroxidation. The antioxidant α-tocopherol acted as a chain breaking antioxidant, whereas the more hydrophilic antioxidant quercetin was marginally chain breaking but more capable to reduction of iron-oxo initiator (inhibition of initiation) (Goupy *et al.*, 2007).

The effect of some polyphenols on deactivation of oxo-ferryl myoglobin in the term of rate constant ($M^{-1}.s^{-1}$) was also reported including that of (-)-epigallo catechin gallate (1170±83), green tea extract (2300±77) (Hu and Skibsted, 2002a), chlorogenate (216±50) (Carlsen *et al.*, 2000), and rutin (105±1) (Jorgensen and Skibsted, 1998). Hu and Skibsted (2002) proposed that

initial formed of the phenoxyl radicals of (-)-epigallo catechin gallate might slowly either disproportionate or reduce other oxo-ferryl myoglobin.

7. Effectiveness of selected extract on inhibition of lipid oxidation in fish slices

The fish slices with thickness of ~8 mm were used for ensuring that the oxygen affinity could be penetrated to whole slice. It was based on the fact that penetration of oxygen from the atmosphere was only 1-4 mm from fish surface (Lawrie, 1974). The initial pH value of tongol tuna flesh (5.95±0.04) was lower than sea bass (6.70±0.01). Although, the initial pH of GE95 solution used as fish soaking media was 6.66±0.01. There was no significant effect on the pH of the fish slices before and just after soaking, this may due to strong buffering capacity of muscle proteins (Kelleher *et al.*, 1992). Effect of soaking treatments on pH of fish slices during storage at 4°C is shown in Table 10. pH of all samples tend to increase as storage time increased particularly in control sample. Additionally, GE95 treated sample could more retard pH changes compared with control sample. This may due to weak acid property of polyphenolic extract.

Table 10 Effect of galangal extract solution (equivalent to 0.20 % w/v gallic acid) treatments on pH value of tongol tuna and sea bass slices during storage at 4°C

Storage	Tongol tuna		Sea bass	
(day)	(control)†	(treat)	(control)	(treat)
0	5.95 ± 0.04^{ax}	5.80 ± 0.02^{ay}	6.70 ± 0.01^{by}	6.68 ± 0.05^{bv}
3	5.84 ± 0.04^{bw}	5.76 ± 0.01^{ay}	6.75 ± 0.01^{cz}	6.74 ± 0.00^{cw}
6	5.83 ± 0.03^{aw}	5.80 ± 0.01^{ay}	6.75 ± 0.00^{cz}	6.66 ± 0.01^{bu}
9	6.82 ± 0.00^{cz}	5.86 ± 0.03^{az}	6.78 ± 0.05^{bz}	6.75 ± 0.00^{bx}
12	6.76 ± 0.03^{by}	5.85 ± 0.04^{az}	6.85 ± 0.01^{cz}	6.79 ± 0.01^{by}
15	6.84 ± 0.01^{bz}	5.87 ± 0.02^{az}	6.88 ± 0.01^{cz}	6.82 ± 0.00^{bz}

† Control: fish samples were soaked in distilled water for the identical condition with the samples

Mean ± SD from triplicate determinations

The different superscripts in the same row (a-c) and column (v-z) denoted the significant differences ($P<0.05$)

Development of LHP in the fish slices during storage at 4°C is shown in Figure 15. It was clear that the preformed LHP in tongol tuna slice (300 μmole LHP/Kg muscle) was significant higher than that of sea bass (85.78 μmole LHP/Kg muscle). The difference in LHP among these slices expressed throughout the experiment. Soaking the slices into the diluted solution of GE95 could maintain the LHP significantly lower than that of the untreated sample throughout the experiment. The development of LHP in the sea bass slices soaking in either diluted GE95 solution or distilled water was lower than 200 μmole LHP/kg sample, excepted the slice treated with distilled water at day 9th of storage. The results suggested that GE95 could retard further development of LHP in the storage tongol tuna muscle. The relative constant amount of LHP in the tongol tuna slices might due to its consequent change to secondary products. In sea bass slices, the development of LHP was negligible thus it effectiveness of GE95 as antioxidant was unclear. LHP is the primary product of lipid oxidation.

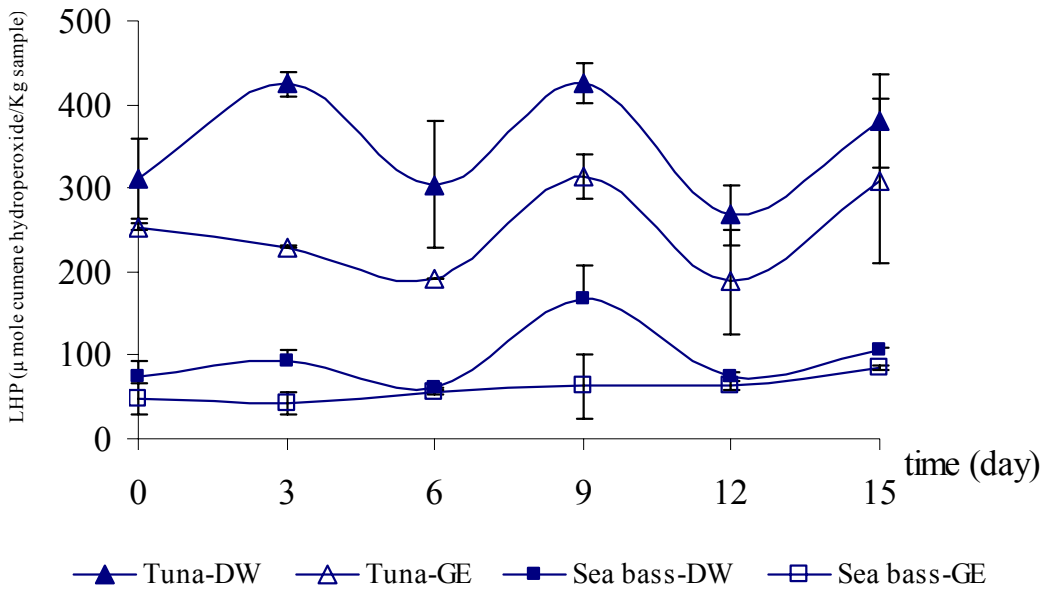


Figure 15 Effect of soaking in diluted galangal extract on lipid hydroperoxides development of tongol tuna and sea bass slices at 4°C. (DW = soaked in distilled water; GE = soked in GE95 solution).

Development of TBARS in the slices is shown in Figure 16. It was found that the lag time before the TBARS of the tongol slices over 7 mg MDA/Kg was after 6 days. It has been proposed that the maximum level of TBARS indicating the good quality of frozen, chilled or stored ice storage fish is 5 mg malonaldehyde/kg, while the fish may be consumed up to the level of 8 mg malonaldehyde/ kg (Schormüller, 1969) that agreed with the results obtained from preliminary study (data not shown). The non significant difference between the lag time of control and GE95 treated sea bass slices suggested that GE95 could not prevent the development of secondary lipid oxidation products.

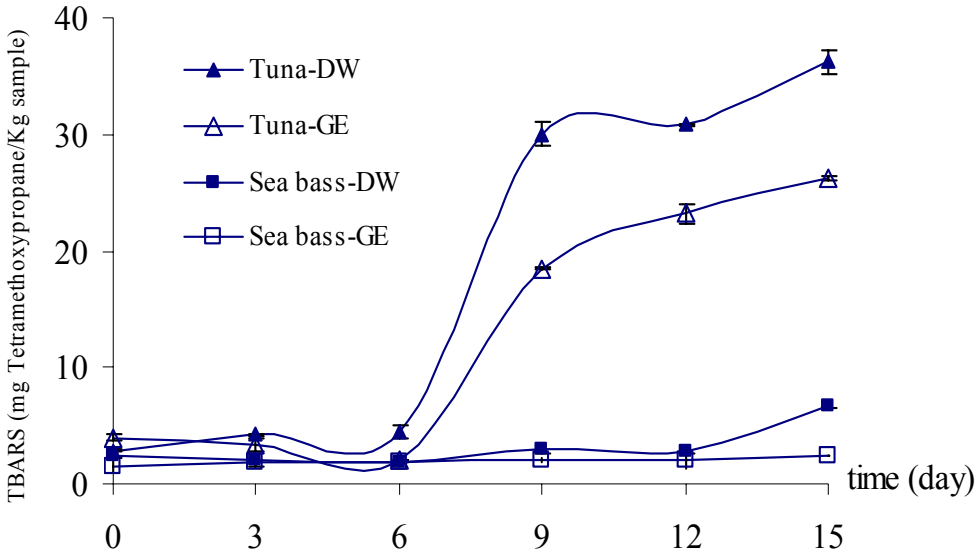


Figure 16 Effect of soaking in diluted galangal extract on TBARS development of tongol tuna and sea bass slices at 4°C. (DW = soaked in distilled water; GE = soked in GE95 solution).

However, the GE95 showed its inhibitory effect on development of TBARS in the sea bass slices with the lag time over 15 days. Whereas, the TBARS value of the untreated slices was closed to 7 mg MDA/Kg at 15th day. This observation pointed that the lack of inhibiting effect of the extract on development of TBARS in tongol slices might associate with high fomed LHP in this sample relative to that of sea bass. On the other hands, the results suggested critical of time by addition of GE95 on its efficacy as antioxidant. Richards *et al*. (2002) found that the preformed LHP was crucial for strong pro-oxidative activities of met-heme proteins. The LHP values related to an effectiveness of oxidizable system that mainly accelerated by heme and non heme-iron (Halliwell and Gutteridge, 1989). The researchers also found that washing the fish fillet obtained

from the fresh fish with antioxidant solution showed inhibition on lipid oxidation (Richards *et al.*, 1998). However, if the fillet was prepared from unfresh fish the minor positive effect was noticed. Apart from low content of preformed LHP, sea bass was white flesh fish having low content of free fatty acids (Kizza *et al.*, 1991) and heme protein (Erkan and Ozden, 2007), as well as filleting the fish was carefully performed to avoid blood fluid contamination. These factors may account for a limited development of lipid oxidation. It is nesesary to note that although, soaking was prolong for 5 min it was not reliable that antioxidative components of GE95 would penetrate throughout the slices.

Discoloration of the slices was monitored by measuring their color values (Table 11). L^* value of sea bass slice was higher than that of tongol slice. This was associated with differenct in heme protein content among these fishes. Changes of L^* value of the slices during storage were likely affected by treatments. In general, lightness of the slices soaked in diluted solution of GE95 was relatively constant throughout the storage. Whereas, the L^* value of the slices soaked in distilled water changed with different patterns depending on fish species. For instance, the L^* value of tongol tuna slice soaked in distilled water increased drastically after storage for 3 days and thereafter gradually increased. Whereas, the L^* value of the sea bass decreased with the storage duration increased. The different patterns may be associated with difference in preformed LHP content, the content and form of heme protein, etc.

The decreasing in L^**values* of sea bass slices during the storage might due to low-temperature browning reactions between sugars or sugar phosphates with proteins. A bright yellow fluorescence arose from reactions involving formaldehyde produced by enzymatic degradation of trimethylamine oxide (Davis and Reece, 1982). Pokorny and Sakurai (2002) mentioned that the different lipid oxidation products, such as free radicals, hydroperoxides and aldehydes reacted with free amine groups of proteins free amino acids formed yellow intermediate products which polymerized into dark brown macromolecules (Pokorny and Sakurai, 2002). In contrast, the alteration of L^* value of tongol slice might due to the reaction between heme proteins and hydrogen sulfide or oxidation products leading to green material at the surface of the slices (Greenwood *et al.*, 1939; O'Sullivan *et al.*, 2003).

Table 11 Effect of galangal extract solution (equivalent to 0.20 % w/v gallic acid) treatments on L^* value of tongol tuna and sea bass slices during storage at 4°C

Storage (day)	Tongol tuna		Sea bass	
	(control) †	(treat)	(control) †	(treat)
0	39.25 ± 3.60^{ax}	39.88 ± 3.24^{ay}	47.57 ± 1.08^{by}	48.68 ± 1.00^{bz}
3	45.68 ± 1.43^{ay}	41.40 ± 2.80^{az}	45.81 ± 0.85^{ay}	48.13 ± 1.93^{az}
6	47.65 ± 0.67^{acyz}	44.17 ± 2.13^{ayz}	40.73 ± 2.14^{ay}	47.78 ± 1.57^{bz}
9	44.93 ± 0.62^{abcyz}	42.97 ± 1.72^{abyz}	47.40 ± 1.35^{cy}	46.06 ± 1.91^{bcz}
12	46.94 ± 0.60^{bcy}	41.22 ± 4.89^{ay}	44.94 ± 1.67^{bcy}	47.47 ± 1.16^{cz}
15	48.70 ± 3.93^{cz}	40.41 ± 3.41^{ay}	41.38 ± 4.46^{abz}	46.21 ± 2.09^{bcz}

† Control: fish samples were soaked in distilled water for the identical condition with the samples

Mean ± SD from triplicate determinations

The different superscripts in the same row (a-c) and column (v-z) denoted the significant differences ($P<0.05$)

Table 12 shows the a^*values of tongol tuna and sea bass slices soaked in distilled water or GE95 extracts. The a^* value of sea bass slices was lower than tongol tuna slices. The a^* value expressed as redness of materials, for meat and fish flesh the major colorants are myoglobin and hemoglobin. Sea bass is a white flesh fish contained lower level of heme-iron than did tongol tuna as reported in Table 1. Blood is more concentrated in dark muscle than light muscle due to greater capillary density in dark muscle compared to light muscle (Mathieu-Costello, 1993).

This may support the fact that a marginal change in a^* value was found in sea bass slices. In tongol tuna slices, there was the trend that the GE95 treated slices had high a*values than that of the untreated samples, excepted the ninth day of storage. It is likely due to the antioxidative activity of GE95 that retarded alteration of heme proteins in the tongol tuna. The decreasing of a* value was fairly correlated to the oxidation of lipid (Richards and Hultin, 2002; Cheah and Abu-Hasim, 2000).

Table 12 Effect of galangal extract solution (equivalent to 0.20 % w/v gallic acid) treatments on *a** value of tongol tuna and sea bass slices during storage at 4°C

Storage	Tongol tuna		Sea bass	
(day)	(control) †	(treat)	(control) †	-3.22±0.18^{az}
0	1.75±0.23^{bz}	2.54±0.78^{bz}	-3.02±0.35^{az}	-3.27±0.15^{az}
3	0.95±0.40^{by}	1.82±0.95^{bz}	-2.89±0.54^{az}	-3.51±0.15^{ayz}
6	0.20±0.89^{by}	1.29±0.33^{by}	-3.03±0.30^{az}	-3.45±0.40^{byz}
9	-1.11±0.32^{cx}	0.77±0.49^{cx}	-4.77±0.67^{ax}	-3.56±0.32^{byz}
12	-1.42±0.72^{cx}	-0.11±0.73^{dwx}	-4.16±0.44^{axy}	-3.58±0.61^{ay}
15	-2.29±0.22^{bw}	-1.12±0.45^{cv}	-3.94±0.08^{ayz}	-3.22±0.18^{az}

† Control: fish samples were soaked in distilled water for the identical condition with the samples

Mean ± SD from triplicate determinations

The different superscripts in the same row (a-c) and column (v-z) denoted the significant differences ($p<0.05$)

Table 13 presents the increasing of *b** values in the slices. At the initial stage the *b** value of tongol tuna slices was much higher than that of sea bass. These results might associate with changes of heme proteins in the samples. The tongol used for the experiment was not as fresh as sea bass. It had been reported that *b** values was affected by the formation of met-/ferryl form of heme (Pearson, 1987). However, there was no insignificant difference in *b** value of tongol tuna or sea bass slice soaked in distilled water and diluted GE95.

However, the experiment did not include the antimicrobial effect of galangal extract that might be interference to the result. Shewan (1971) reported that microbial could use the TMAO to produce TMA that might interact with heme proteins thus giving the green pigments. This may be another cause of discoloration of tongol slices during storage.

Table 13 Effect of galangal extract solution (equivalent to 0.20 % w/v gallic acid) treatments on *b** value of tongol tuna and sea bass slices during storage at 4°C

Storage	Tongol tuna		Sea bass	
(day)	(control) †	(treat)	(control) †	(treat)
0	7.83 ± 0.39^{bw}	7.59 ± 1.27^{bx}	-2.14 ± 1.29^{ax}	-0.74 ± 1.49^{ay}
3	9.10 ± 0.37^{bx}	10.82 ± 0.55^{byz}	-2.21 ± 0.98^{ax}	-1.22 ± 0.78^{ay}
6	10.00 ± 0.22^{bx}	10.13 ± 0.57^{by}	0.68 ± 0.65^{ay}	0.06 ± 0.66^{ay}
9	11.39 ± 0.25^{by}	11.15 ± 1.00^{byz}	0.02 ± 1.60^{ay}	1.49 ± 3.73^{ayz}
12	11.91 ± 1.18^{byz}	11.84 ± 1.14^{bz}	1.05 ± 1.87^{ay}	1.12 ± 1.13^{ayz}
15	12.97 ± 1.31^{bz}	11.02 ± 0.38^{byz}	4.26 ± 0.87^{az}	3.57 ± 2.08^{az}

† Control: fish samples were soaked in distilled water for the identical condition with the samples

Mean ± SD from triplicate determinations

The different superscripts in the same row (a-c) and column (v-z) denoted the significant differences ($P<0.05$)

CHAPTER 4

CONCLUSIONS

Summary I ***Sea bass hemoglobin (de)oxygenation***

Sea bass hemoglobin was rapidly (de)oxygenated at the pH 6.2 that showed a likely honorary behavior. Whereas, at pH 7.0 the sea bass hemoglobin was dominated in oxygenated form. The ATP was a lesser potent to deoxygenation of sea bass hemoglobin than effect of pH.

Summary II ***Hemoglobin-mediated lipid oxidation***

The pro-oxidative property of sea bass hemoglobin was potent when the pH was 6.2 and physiological pH. Whereas, the lipid oxidation was retarded at the pH 7.0. The initial level of preformed LHP was a factor enhancing lipid oxidation in fish muscle when postmortem age increased. Therefore, the presence of hemoglobin was likely synergized with the preformed LHP to acceleration of lipid oxidation.

Summary III ***Antioxidative properties of galangal and lemon grass***

The polyphenolic contents of galangal and lemon grass were dependent on the ethanol concentration. The DPPH radical scavenging activity of galangal and lemon grass depend on the ethanol concentration. Whereas, the chelating activity of galangal extract was independent to the ethanol concentration. Galangal was an interested source of natural chelating agent. The antioxidative activities of galangal and lemon grass extracts were ethanolic concentration dependent. The GE95 showed the antioxidant activities but less effectiveness in sea bass muscle system. Moreover, the GE95 could extent the oxy-form of hemoglobin resulting in lowering a pro-oxidative activity of hemoglobin.

Summary IV ***Preservation of fish muscle by GE95***

The soaking of fish slices in the diluted solution of GE95 could retard the lipid oxidation, especially in the tongol tuna slices. The GE95 showed a major role as a primary antioxidant. However, it had less effectiveness in sea bass.

Future works

1. The study on antioxidative properties of galangal and lemon grass extracts against acceleration of lipid oxidation by hemoglobin and myoglobin.
2. The study on the antioxidative properties of other natural sources for inhibition of the hemoglobin-mediated lipid oxidation.
3. The effect of some natural antioxidants on preservation of blood water.

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APPENDIX

Analytical Methods

1. Determination of moisture content (AOAC, 2000)

Method

1. Dry the empty moisture can and lid in the oven at 105°C for 3 h and transfer to desiccator to cool. Weight the empty can and lid.
2. Weight about 3 g of sample to the moisture can. Spread the sample to the uniformity.
3. Place the can with the sample in the oven. Dry for 3 h at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the can and its dried sample.

Calculation

$$Moisture\ (\%) = \frac{(w_1 - w_2)}{w_1} \times 100$$

Where: w_1 = weight (g) of sample before drying

w_2 = weight (g) of sample after drying

2. Determination of protein content (AOAC,2000)

Reagents

- 0.2 N HCl solution
- 4% H_3BO_3
- 40 % NaOH solution
- Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)
- Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of copper sulphate ($CuSO_4$)
- Sulfuric acid (H_2SO_4)

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjedahl catalyst and 200 ml of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently until frothing ceases. Boil briskly until the solution becomes clear.
4. Cool and add 60 ml of distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 drops of indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

$$\text{Protein}\,(\%) = \frac{(A - B) \times N \times 1.4007 \times 6.25}{w}$$

Where: A = volume (ml) of 0.2 N HCl used sample titration

B = volume (ml) of 0.2 N HCl used in blank titration

N = volume (ml) of 0.2 N HCl used sample titration

w = weight (g) of sample after drying

14.07 = atomic weight of nitrogen

6.25 = the protein-nitrogen conversion factor for fish and its by-products

3. Determination of ash content (AOAC, 2000)

Method

1. Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.
4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.

5. Heat at 550°C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

$$Ash\ (\%) = \frac{Weight\ of\ ash}{Weight\ of\ sample} \times 100$$

4. Determination of lipid content (Bligh and Dyer, 1959)

Reagents

- Chloroform
- Methanol

Method

1. Homogenize the sample (20 g) with 16 ml of distilled water, 40 ml of chloroform and 80 ml of methanol at the speed of 9,500 rpm for 1 min at 4°C.
2. Add 40 ml of chloroform and homogenize for 30 sec.
3. Add 40 ml of distilled water and homogenize again for 30 sec.
4. After centrifugation of the homogenate at 2,000 rpm at 4°C for 20 min, transfer the supernatant into a seperatory funnel and allow to separate.
5. Determine lipid content gravimetrically by measuring triplicate aliquots of the chloroform layer into tared containers, evaporate the solvent and weigh.
6. Calculate the lipid content.

5. Determination of heme-iron content (Hornsey, 1956)

Reagents

- Acetone
- Hydrochloric acid

Method

1. Weigh 10 g of ground sample into 50 ml centrifuge tubes.
2. Added 20 ml of acid–acetone mixture (40 ml of acetone, 9 ml of water, and 1 ml of concentrated hydrochloric acid).

3. Homogenize the sample for 30 s with a blender.
4. Add 20 ml of acid–acetone mixture and the samples mix thoroughly, and kept in the dark for 1 h.
5. Centrifuged at 2200*g* for 10 min. Filter the supernatant through filters (Whatman GF/A) and measure the absorbance at 640 nm against a reagent blank.
6. Measure the absorbance of the filtrate at 640 nm, and calculated the heme iron content by using a molar extinction coefficient of 4800 M^{-1} cm^{-1}.

6. Determination of non heme-iron content (Ahn and Nam, 2004)

Reagents

- 10% ammonium acetate
- 2% ascorbic acid in 0.2 M HCl
- Citrate-phosphate buffer (pH 5.5)
- Ferrozine colour reagent
- Iron standard

Method

1. Transfer meat sample(5 g) or 3.74 ml iron standard solution (0, 1, 2, 3 or 4 ppm) to a 50 ml poly-propylene centrifuge tube and add 15.0 ml of 0.10 M citrate-phosphate buffer at pH 5.50.
2. Homogenize the suspension for 30 s at 13 500 rpm.
3. Rinsed twice with 250 ml citrate-phosphate buffer for 3 min at 13 500 rpm, to minimise release of iron during homogenisation of the samples.
4. Homogenate (1.5 g) was transferred to a 5 ml sample tube, treat with 0.50 ml of 2% ascorbic acid in 0.2 M HCl and incubate at room temperature for 15 min.
5. Add 1 ml of 11.3% trichloroacetic acid the sample and mixed thoroughly, then transfer 2 ml sample to a centrifuge tube and centrifuged for 10 min at 20 000 *g*.
6. Mix 1 ml of the clear supernatant with 0.40 ml of 10% ammonium acetate and 0.10 ml of ferrozine colour reagent.
7. The mixture was filtered through a filter pore size of 0.2 μm, transfer to a disposable semi-micro cuvette and read the absorbance at 562 nm.

8. Calculate the concentration of non-heme iron from a standard curve.

7. Hemolysate preparation (Fyhn *et al*., 1979)

Reagents

- 1.7% NaCl in 1 mM Tris, pH 8.0
- 1 mM Tris, pH 8.0
- 1 M NaCl

Method

1. Add 4× volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0 to heparinized blood and centrifuged at 700× ***g*** for 10 min, 4 °C.
2. Remove the supernatant and wash the pellet (the red blood cells) by suspending 3 times in 10× volumes of the above buffer.
3. Add 3× volumes of 1 mM Tris, pH 8.0 to the pellet and incubated for 1 h, on ice.
4. Add 1/10× volume of 1 M NaCl to aid in stromal removal before ultracentrifugation at 28,000 ***g*** for 15 min at 4 °C.
5. Kept the hemoglobin solutions in the supernatant and stored at −80 °C prior to use.

8. Determination of hemoglobin content in diluted concentration (Richards *et al*., 2002)

Reagents

- 50 mM Tris buffer pH 8.0
- Carbon monoxide gas
- Sodium dithionite

Method

1. Dilute the concentrated hemoglobin solutions were diluted 500 times with 50 mM Tris buffer pH 8.0.
2. Add around 1 mg of sodium dithionite to 1.5 ml of the hemolysate and mixed in a cuvette then bubble carbon monoxide gas into the samples for 30 s.
3. Scan the spectrum from 440 to 400 nm (Soret band) against a blank that contained only buffer.
4. Record the peak at 420 nm and use the bovine hemoglobin as a standard.

9. Determination of hemoglobin content in concentrated blood (Drabskin and Austin, 1935)

Reagents

- $K_3Fe(CN)_6$ 200 mg/L
- KCN 50 mg/L
- $NaHCO_3$ 1 g/L

Method

1. Add 800 µL of Drabkin's reagent ($K_3Fe(CN)_6$ 200 mg/L, KCN 50 mg/L, $NaHCO_3$ 1 g/L, pH 8.6) to a 200 µL aliquot and allow to stand for 15 minutes.
2. Measure the O.D. at 550 nm (This reaction converts hemoglobin to cyanoMetHb, which has an absorbance peak at 540 nm, and whose concentration can then be assessed by the OD of the solution at ≈550 nm wavelength).
3. Use bovine erythrocyte hemoglobin as standard.

10. Determination of hemoglobin oxygenation (Richards and Hultin, 2000)

Reagent

- 50 mM Tris buffer pH 8.0

Method

1. Scan the spectrum from 630 to 500 nm of the solutions containing hemoglobin and run at atmospheric conditions. The blank contained only buffer.
2. Calculate the absorbance at the peak (575 nm) minus the absorbance at the valley (560 nm).

11. Washing of fish minced (Richards and Hultin, 2000)

Reagent

- 50 mM sodium phosphate (pH 6.0)

Method

1. Grind the white muscle in a mincer at 4 °C.
2. Washed the minced once in distilled water at a 1:3 mince to water ratio (w:w) for 2 min.
3. Allow the mixture to stand for 15 min before dewatering with fiberglass screen.
4. Wash the minced with three volumes of 50 mM sodium phosphate (pH 6.0), dewatered and wash a final time in 50 mM sodium phosphate (pH 6.0).
5. Homogenized the slurry.
6. Centrifuge at 15,000*g* for 25 min at 4 °C and stored at 4°C until use.

12. Determination of lipid hydroperoxide (LHP) value (Richards *et al*., 2002)

Reagents

- Chloroform/ methanol (1:1)
- 0.5% NaCl
- 4.38 M ammonium thiocyanate
- 18 mM iron (II) chloride
- Cumene hydroperoxide

Method

1. Weigh the muscle between 400-500 g and homogenize in 5 ml of chloroform/ methanol (1:1) for 30 s.
2. Rinse the polytron with 5 ml of solvent for 30 s.
3. Add 3 ml of 0.5% NaCl and vigorous mix with a Vortex for 30 s.
4. Centrifuge for 10 min (4 °C and 700 g) to separate the mixture into two phases.
5. Add 1.33 ml of ice cold chloroform / methanol (1:1) to 2 ml of the lower phase and mix briefly.
6. Add 25 µl of ammonium thiocyanate (4.38 M) and 25 µl of iron (II) chloride (18 mM).
7. Incubate the reaction mixtures at room temperature for 20 min.
8. Measure the absorbance at 500 nm
9. Use cumene hydroperoxide for standard curve construction.

13. Determination of thiobarbituric reactive substances (TBARS) (Beuge and Aust, 1978)

Reagents

- Thiobarbituric acid
- Hydrochloric acid
- Trichloro acetic acid
- Tetramethoxy propane

Method

1. Add 5 ml of the TBARS mixture to ~ 150-400 mg of sample and incubate at 95 °C for 20 min.
2. Centrifuge (2500× ***g*** for 10 min) then measure the absorbance of the supernatant at 532 nm.
3. Calculate the TBARS from a standard curve by using tetramethoxy propane.

14. Determination of polypolyphenolic content (Zhu *et al.*, 2002)

Reagents

- 0.1 N Folin–Ciocalteu reagent
- 30% sodium carbonate
- Gallic acid
- Catechin

Method

1. Add 0.5 ml of 0.1 N Folin–Ciocalteu reagent to 0.5 ml of sample.
2. After 5 min, add 2 ml of 30% sodium carbonate into the mixture.
3. Incubate the mixture at ambient temperature in darkly chamber.
4. Determine the absorbance at 760 nm.
5. Calculate the polypolyphenolic content by using gallic acid or catechin as standard.

15. Determination of DPPH radical scavenging activity (Xu *et al*., 2004; Yokozawa *et al*., 1998)

Reagent

- 43 μM of DPPH in methanol
- 50% aqueous ethanol (v/v)

Method

1. Add 100 μl of 43 μM of DPPH in methanol to 100 μl the serial diluted extract.
2. Incubate at 22–24 °C for 30 min in an opaque chamber
3. Measure the absorbance at 515 nm.
4. Use Methanolic DPPH and 50% aqueous ethanol (v/v) as control.

Calculation

$$\% \text{ Inhibition} = \frac{(O.D._{control} - O.D._{sample})}{O.D._{control}} \times 100$$

16. Determination of chelating activity (Hsu *et al*., 2006)

Reagent

- 2 mM $FeCl_2$
- 5 mM ferrozine in methanol
- EDTA

Method

1. Add 50 μl $FeCl_2$ (2 mM) and 1.6 ml deionised water to 500 μl of extract.
2. Shake the mixture vigorously and stand at room temperature for 5 min.
3. Add 100 μl of ferrozine (5 mM in methanol), mix and allow stand for 5 min to complex the residual Fe^{2+}.
4. Measure the absorbance at 562 nm against a blank.
5. Use EDTA as the control.
6. The chelating activity of the extract for Fe^{2+} was calculated as

$$\% \text{ Chelating} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 was the absorbance of the blank (without extract)

A_1 was the absorbance in the presence of the extract

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